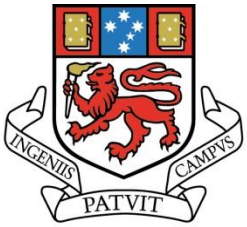

UNIVERSITY
OF TASMANIA

HOST RESPONSE TO AMOEBIC GILL DISEASE IN ATLANTIC SALMON AND BLOOD FLUKE INFECTION IN PACIFIC BLUEFIN TUNA

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Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

June, 2016

DECLARATIONS BY THE AUTHOR

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STATEMENT OF CO-AUTHORSHIP

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Paper 1 (Chapter 2)

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Candidate was the primary author and performed all laboratory analyses. Author 1, author 2 and author 4 contributed to the idea, its formalisation and development, refinement, and presentation. Author 2 and 3 conceived, designed and performed the experiment. Candidate, author 2 and 3 collected samples. Author 2 contributed to data analyses.

Paper 2 (Chapter 3)

Pennacchi Y., Adams M., Nowak B., and Bridle A. (2016). Immune gene expression in the gills of Atlantic salmon (*Salmo salar* L.) following experimental reinfection with *Neoparamoeba perurans*. Aquaculture (Submitted).

Candidate was the primary author and performed all molecular and image analyses. Author 1, author 2 and author 5 contributed to the idea, its formalisation and development, refinement, and presentation. Author 5 conceived, designed and performed the experiment. Candidate and author 5 collected samples. Author 2 contributed to molecular data analyses. Author 5 contributed to histological analyses and histological data analyses.

Paper 3 (Chapter 4)

Pennacchi Y., Shirakashi S., Nowak B., and Bridle A. (2016). Immune reactivity in early life stages of sea-cage cultured Pacific bluefin tuna naturally infected with blood flukes from genus *Cardicola*. Fish and Shellfish Immunology (Submitted).

Candidate was the primary author and performed all molecular, histological and data analysis. Author 1, 2 and 6 contributed to the idea, its formalisation and development, refinement, and presentation. Candidate and author 6 designed sampling strategy and collected samples.

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ABSTRACT

Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, and *Cardicola* spp. infection represent a great concern for the sea-cage culture of Atlantic salmon in Australia, and Pacific bluefin tuna (PBT) in Japan. These fish parasitoses have been linked to mortality of the affected individuals if treatment is not provided. To date the only treatments used by industries to mitigate the outbreaks of these parasitic diseases and avoid mortality are the freshwater bath and the hydrogen peroxide bath (Northern hemisphere) of the AGD-affected Atlantic salmon stocks and the administration of the antihelminthic drug praziquantel (PZQ) to bluefin tuna infected by *Cardicola* spp. Several studies have been carried out on AGD and *Cardicola* spp. infection, nevertheless little is known about the host immune response to these infectious diseases. To deepen our knowledge of the fish immune response to AGD and *Cardicola* spp. infection is of utmost importance to contribute to the development of immune-based health strategies or other practical solutions to improve the fish health in mariculture.

In this thesis, to provide a better understanding of the host inflammatory and immune response to *N. perurans* and *Cardicola* spp., several genes were investigated at the transcriptional level by using quantitative real-time PCR. In particular, research focused on the fish gills as this organ has been linked to host mortality due to lack of oxygen in both parasitic diseases. Other techniques used to assess and describe the severity of pathology in the fish gills were routine histology for both AGD and *Cardicola* spp. infected individuals and gross gill score and image analysis for AGD-affected fish. Furthermore, a quantitative molecular technique was used to determine the species-specific relative prevalence of *Cardicola* spp. DNA in the fish organs and describe the natural infection timeline.

In Chapter 2 the characterisation of pro-inflammatory (IL-1β and TNF-α) and immune (TCR-α chain, CD8, CD4, MHC-IIα, MHC-I, IgM and IgT) related genes at the mRNA level in the gills of AGD-affected Atlantic salmon was performed at 10 days post-exposure to *N. perurans.* A significant

up-regulation of the mRNA expression was detected in the AGD-affected gills, suggesting that the parasite elicits a classical inflammatory response in the host and most importantly that the immune gene expression within AGD-lesions misrepresents the cellular immune response in the gills during AGD.

Chapter 3 focused on investigating the Atlantic salmon immune response to *N. perurans* following reinfection. The targeted pro-inflammatory and immune-related genes were IL-1β, TCR-α chain, CD8, CD4, MHC-IIα, MHC-I, IgM and IgT. Gross gill score was used to assess the AGD-severity during the trial and determine the fish categories for gene expression. Histopathology and image analysis were used to further assess the AGD-severity. Overall the expression at the mRNA level of the immune-related genes analysed showed little change in AGD-affected gills of experimentally reinfected fish.

In Chapter 4 the inflammatory and immune response in the gills of cultured juvenile PBT during early infection with *Cardicola* spp. at the transcriptional level was described by using a quantitative real-time PCR. Furthermore, *C. orientalis* and *C. opisthorchis* DNA within host gills and heart was quantitatively detected using qPCR. An increase of immune-related genes, namely IgM, MHC-I, TCR-β and IL-1β was observed in the PBT gills infected by *Cardicola* spp. Most notably, IgM and MHC-I transcription was strongly up-regulated in gill samples infected by *C. orientalis* relative to uninfected fish but not by *C. opisthorchis*. *Cardicola*-specific DNA was first detected in the host 14 days post transfer (DPT) to sea-cages and 55 days earlier than the first observation of parasite life stages with microscopy and histology. PZQ treatment did not have an immediate effect on parasite DNA presence in the host. A reduction in *Cardicola* spp. DNA was detected 24 days post PZQ in the host heart. Altogether these findings indicate the involvement of an immune reaction in the host gills during early *Cardicola* spp. infection although the response was not effective at clearing or mitigating the primary infection.

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EXPLANATORY NOTE CONCERNING THESIS STRUCTURE

Chapters 2, 3 and 4 of this thesis have independently been published (or submitted) as journal articles. Therefore, some textual and reference overlap occurs between these chapters (especially between chapters 2 and 3). Chapter 1 of this thesis is written as a general introduction and review of relevant topics needed to establish the experimental rationale of the subsequent research chapters. In Chapter 5, as general discussion, are discussed the main findings of the research, their implications and limitations offering also some recommendations for future work. The Aquaculture referencing style has been adopted for this thesis; however, the orthography is consistent with the commonwealth countries of Britain. The bibliography section is presented at the end of the thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

1.1. Fish immunity

The immune response is a set of defensive mechanisms of an organism that is based on the recognition of non-self, such as a pathogen. However, in some autoimmune diseases the immune system elicits an immune response to self. Understanding these mechanisms is of utmost importance in order to properly manage the health of a fish species in aquaculture. Evolutionarily, fish are the most ancient and heterogeneous group of vertebrates representing a turning point between innate immunity and the emergence of adaptive immunity (Rauta et al., 2012).

Fish immune organs (thymus, kidney and spleen) are homologous to those found in mammals (Rauta et al., 2012). The kidney, divided into anterior/head and posterior, represents the bone marrow homolog and in contrast to higher vertebrates, it is the main immune organ responsible for antigen processing, immunoglobulin M (IgM) and memory cell production (Rauta et al., 2012). In fish early stages the kidney represents the largest site of haematopoiesis (Zapata et al., 2006). For instance, alevins of *Oncorhynchus mykiss*, rainbow trout, possess a well-developed kidney producing red blood cells and granulocytes (Uribe et al., 2011). In fish, the spleen similarly to mammals, contains IgM+ B cells and plays an important role in haematopoiesis, antigen degradation and antibody production (Rauta et al., 2012). The thymus is located near the gill cavity in teleosts, and it is mainly a site of T cell production (Uribe et al., 2011). Lipopolysaccharide (LPS)-sensitive IgM+ B cell populations can also be found in the blood of teleosts (Bromage et al., 2004; Zwollo, 2011).

The mucosae of teleosts are also active immunological sites and constitute the mucosa-associated lymphoid tissue (MALT) which can be sub-divided into three components according to their anatomical location: gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), and gill-associated lymphoid tissue (GIALT) (Salinas et al., 2011). Leukocytes such as T cells, B cells, plasma cells, macrophages, and granulocytes can be found in all teleosts' MALTs (Salinas et al., 2011). Another more recent described MALT is the interbranchial lymphoid tissue

(ILT) which contains numerous T cells embedded in a meshwork of epithelial cells with scattered B cells (Haugarvoll et al., 2008; Koppang et al., 2010).

Similar to all jawed vertebrates the fish immune system is composed of different layers. An outer physical barrier (e.g. mucus, skin, gills and intestine) which offers protection against various pathogens (e.g. bacteria, viruses, fungi and parasites) (Boyton and Openshaw, 2002; Bruce et al., 2002); an innate immune defence or non-specific response that protects the organism in case the pathogen breaks the physical barrier (Litman et al., 2005) and a specific response or adaptive immune defence if the pathogen evades the innate defence (Rauta et al., 2012). However, these three components of the fish immune system (i.e. physical barrier, innate and adaptive immune defence) are integrated into a multilevel network (Flajnik and Du Pasquier, 2004). In fact, the innate components are secreted into the mucus and work before the physical barriers are broken and the innate functions are necessary for a properly functional adaptive response.

Innate immunity in fish plays an important protective role against pathogens and can be sub-divided into three parts: the epithelial/mucosal barrier, the humoral and cellular components (Uribe et al., 2011). Fish scales, skin and its mucous surfaces represent a first physical barrier against pathogens (Ellis, 2001). In particular, the fish mucus is very efficient at trapping pathogens and it also contains immune parameters such as lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and IgM/IgT (Aranishi and Nakane, 1997; Boshra et al., 2006; Rombout et al., 1993; Saurabh and Sahoo, 2008). The humoral component is mainly expressed as soluble molecules of plasma and includes antimicrobial peptides, the complement system (classical, alternative and lectin), lysozyme and natural antibodies (Magnadottir, 2006; Whyte, 2007). The key cell types of the innate cellular component are the phagocytic cells (monocytes/macrophages and granulocytes) and the non-specific cytotoxic cells (NCC) (Evans et al., 2001; Froystad et al., 1998). The NCC are able to eliminate various target cells such as tumour cells, virally transformed cells and protozoan parasites (Whyte, 2007). Furthermore, epithelial and dendritic-like cells also play a role in the fish innate immunity (Dalmo et al., 1996; Ganassin and Bols, 1996; Lovy et al., 2006; Ohta et al., 2004; Press et

al., 1994). Importantly, the non-specific cellular response allows the rapid mobilization of large cell populations either at local and/or systemic level following antigen stimulation (Whyte, 2007).

Differently from the innate immune system, that recognises and defends the fish against the non-self in a non-specific way, the adaptive immune system, activated by elements of the innate response (e.g. antibacterial peptides, lysozyme, lectins, acute phase proteins and complement system), is able to produce memory cells and specific cell-receptors (e.g. T-cell receptors) that specifically recognise and eliminate the pathogen (Ellis, 2001; Rauta et al., 2012; Swain, 2006). The genetic elements that are fundamental for adaptive/combinatorial immunity are present in all the jawed vertebrates (Marchalonis et al., 2006). The combinatorial immune system (CIS) includes the antigen-recognising lymphocytes, immunoglobulins (antibodies and immunoglobulin-family T-cell receptors), major histocompatibility complex (MHC) products, and recombination-activating (RAG) 1 and 2 genes (Rauta et al., 2012). Fish and mammals have a similar overall shape of the adaptive immune elements and recombination mechanisms, which is the cause of variability in TCRs and immunoglobulins (Du Pasquier, 2001). Furthermore, teleosts have been shown to possess T- and B-lymphocytes analogous to their mammalian counterparts (Rauta et al., 2012).

In teleosts, the antibody response is mounted after immunisation with specific antigens (Buonocore and Scapigliati, 2008). The antigen receptors are distributed on T and B cells allowing the clonal selection of pathogen-specific receptors that is fundamental for the development of the immunological memory (Alvarez-Pellitero, 2008a). There are two types of T cells that express antigen receptors on their cell surface: Th cells which use as co-receptors CD4 and cytotoxic T cells that use CD8 (Alvarez-Pellitero, 2008a). These cells recognise the antigen peptides that are bound respectively to MHC-II and –I, a critical step to initiate the adaptive immune response (Alvarez-Pellitero, 2008a). Both MHC-I and –II have been described in teleosts and their role is to display antigens to T cells (MHC-I for intracellular pathogens and MHC-II for extracellular pathogens) (Alvarez-Pellitero, 2008a). B cells can recognise almost every antigen and express B-cell receptors or surface immunoglobulin receptors. The immunoglobulins or antibodies are the main indicators of

adaptive immunity and can recognise a large number of different antigens (Rauta et al., 2012). In teleosts, the main immunoglobulin is a tetramer of the IgM class (Acton et al., 1971) that can be found in serum and secretions (e.g. cutaneous and gut mucus) (Alvarez-Pellitero, 2008a). Furthermore, the fish antibody response and serum concentration of IgM depends on the fish species (Alvarez-Pellitero, 2008a). For instance, salmonids can produce a large amount of specific antibodies to various antigens (Alvarez-Pellitero, 2008a) whereas cod, *Gadus morhua*, has no or very low antibody production following antigen encounter (Solem and Stenvik, 2006). IgD, IgT and IgZ have also been demonstrated in fish although their functional characterisation has not been completed yet (Hansen et al., 2005; Ohta and Flajnik, 2006; Randelli et al., 2008). A study carried out in 2010 on the host response against *Ceratomyxa shasta*, an intestinal parasite, revealed that IgT in rainbow trout is specialised in gut mucosal immune responses, while IgM appears to be more involved in systemic immunity (Zhang et al., 2010). A more recent study on the ontogenetic development of the immune system of rainbow trout showed that IgT is also involved in gill mucosal immunity (Heinecke et al., 2014). The mucosal association of IgT was further confirmed by significant up-regulation of IgT and IgD transcripts in rainbow trout gills immunised with an attenuated *Flavobacterium psychrophilum* strain by the immersion route (Makesh et al., 2015). Similarly, the same authors (Makesh et al., 2015) reported an increase of the IgT and IgD transcripts in the fish intestine immunised by anal intubation route.

1.1.1. Inflammatory response

Inflammation is an organism's protective non-specific response to the non-self (e.g. pathogens, foreign particles) or an injury caused by physical trauma and it involves a well-organized humoral and cellular cascade within the injured tissue (Medzhitov, 2008) (for a detailed description of the inflammatory cascade see **Fig. 1.1.**). However, in some autoimmune diseases the inflammatory response can also be directed to the self. The effects of an inflammatory reaction on the organism can be both local, contained at the injury site, and systemic involving the whole body (Ashley et al.,

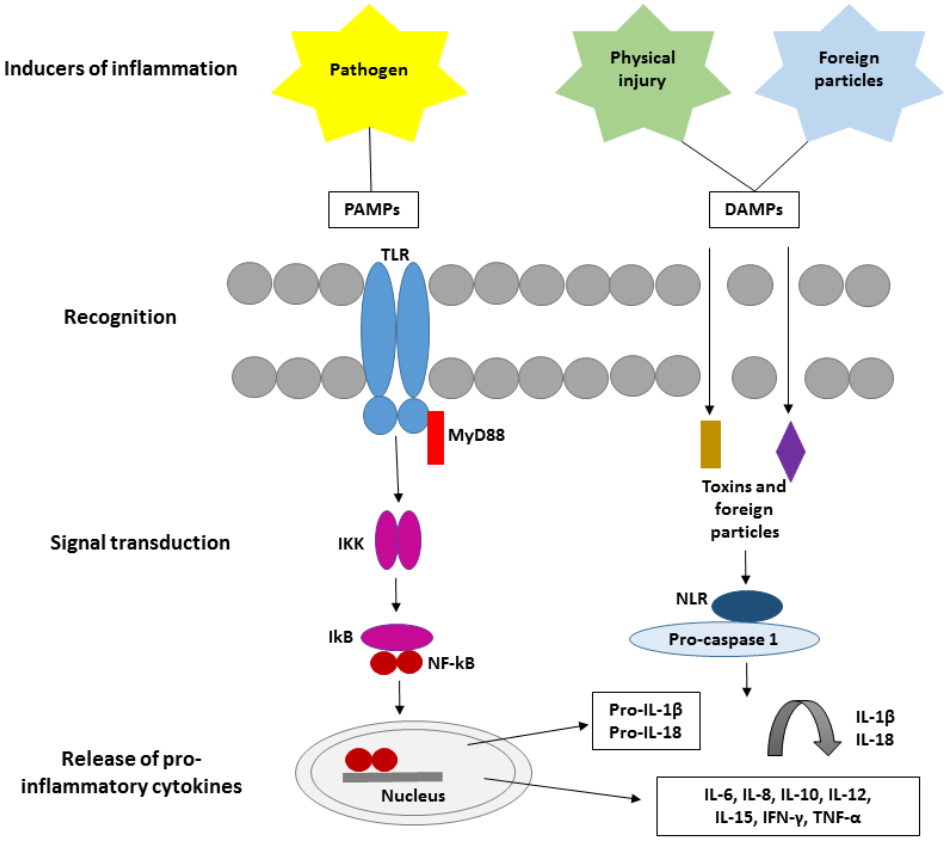

Fig. 1.1. Inflammatory cascade. Pathogens, physical injuries and foreign particles elicit an inflammatory response. Innate transmembrane Toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs) have an essential role in inflammation binding respectively to pathogen associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs). TLRs activate a MyD88-dependant signal transduction pathway which affects the phosphorylation of the inhibitory IκB protein by a kinase complex (IKK). NF-κB is released from IκB and moves to the cell nucleus where it binds to target inflammatory genes. Here various pro-inflammatory cytokines and chemokines are released to stimulate effector functions of the inflammatory response. NLRs are also responsible to activate the converting enzyme caspase-1 that transforms the cytokines into active forms able to trigger inflammation following their release from the cell. Modified from Ashley et al., 2012.

2012). The initial stages of an inflammatory process are defined as acute inflammation and involve the coordinated delivery of blood components, such as plasma and leukocytes, to the infection or injury site (Kumar et al., 2012). If the acute response is unable to eliminate the pathogen or foreign particles, the inflammation shifts to a chronic state which involves pathological consequences such as the formation of granulomas (Kumar et al., 2012). In particular, during a parasitic infection granulomas are the result of an unsuccessful attempt by macrophages to envelop and eliminate the pathogen and its products from the host body (Kumar et al., 2012). This happens due to the fact that multicellular parasites are too large, compared to viruses and bacteria, to be directly eliminated by individual cells (Allen and Wynn, 2011). A cellular response in some cases associated with a

granulomatous condition has been also described in various fish parasitoses. For instance, an inflammatory cell mediated response has been observed in the gills of *Salmo salar* infected by *Neoparamoeba perurans* (see Adams and Nowak, 2003), in the blood of salmonids parasitised by the haemoflagellate *Cryptobia salmositica* (see Woo, 2003), in the gills of *Thunnus orientalis* infected by blood flukes of the genus *Cardicola* (see Shirakashi et al., 2012b), and in salmonid spironucleosis (Alvarez-Pellitero, 2008b).

Cytokines are a group of small secreted proteins with an important role as modulators of the immune response. Most notably some cytokines are fundamental during the inflammatory cascade (**Fig. 1.1.**) as responsible for triggering inflammation through the recruitment of effector cells (e.g. monocytes and neutrophils) to the site of infection or injury (Secombes et al., 2001). In particular, Secombes et al., 2001 showed that fish have various functionally active pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. Other regulatory cytokines found in fish include interleukins ascribed to the IL family (Bei et al., 2006; Igawa et al., 2006; Randelli et al., 2008), 3 isoforms of tumour growth factor (TGF)-β (Randelli et al., 2008), interferon regulatory factor (IRF)-1 (Ordas et al., 2006) and interferons (IFN) (Altmann et al., 2003; Robertsen, 2006). In particular, IL-1β, mainly produced by macrophages in fish has been shown to mediate the inflammatory response to infection, to directly affect the hypothalamic-pituitary-interrenal axis function and stimulate the secretion of cortisol (Castro et al., 2011; Holland et al., 2002).

1.2. Aquaculture

The term aquaculture refers to the farming of aquatic organisms (e.g. finfishes, crustaceans, molluscs, amphibians and plants) that requires some kind of intervention aimed to improve the rearing techniques in order to increase the production. According to the most recent report of the Food and Agriculture Organization of the United Nations, the world consumption of seafood per capita has significantly increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). To

face this large and continuous demand for seafood worldwide, especially in Asian countries, the aquaculture production doubled from approximately 35 million tonnes in the 1960s to 66.6 million tonnes in 2012 (FAO, 2014). Asia is the leading producing continent accounting for 88% of the total world aquaculture production (**Fig. 1.2.**) (FAO, 2014). Although mariculture fish production represents only 12.6% of the total aquaculture fish production, it accounts for almost 27% of the total value of all farmed fish species (FAO, 2014). This is due to the fact that mariculture produces fish species (e.g. salmonids) of higher economic value compared to inland aquaculture (FAO, 2014).

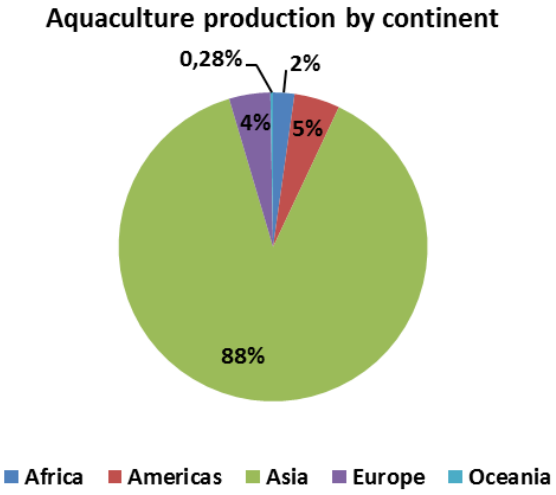

Fig. 1.2. World aquaculture production by continent (excluded algae) (FAO, 2014).

1.3. Parasitic diseases of fish farmed in sea cages

1.3.1. Overview of sea-cage culture

Historically the first attempts to farm fish in sea-cages dates back to 200 years ago in Japan where Japanese fishermen used the net-cages mainly to ranch sardines and anchovies for short periods of time (Takashima and Arimoto, 2000). However, the sea-cage culture for commercial purposes started only during the early 1950s in Japan where researchers at Kinki University successfully cultured the Japanese amberjack (*Seriola quinqueradiata*) (Chua and Tech, 2002). To date, several fish species are farmed in sea-cages of various designs and sizes in different countries worldwide

(Chua and Tech, 2002). The rapid growth of sea-cage farming is mainly ascribed to the development of successful breeding techniques, specific diets, supporting industries (e.g. feed, processing, net manufacturing, packing industries) and culture systems (Chua and Tech, 2002). Altogether the lack or limited access to coastline sites ideal for sea-cage farming, the marine environmental issues associated with intensive fish farming (e.g. build-up of organic matter), and the conflicts with sea tourism, forced some industries to start an offshore cage culture (Chua and Tech, 2002; Lisac, 1991). Both inshore and offshore cage culture present several relevant advantages compared to other culture types, such as an optimization of carrying capacity per unit area, easier construction of the facilities and higher economical profit (Beveridge, 1983; Chua and Tech, 2002). Nevertheless, cage culture has some disadvantages, such as a susceptibility to natural hazards (e.g. tides, storms), seawater pollution and most importantly to an increased exposure to different pathogens and parasites which are more difficult to treat in cages (Chua and Tech, 2002; Nowak, 2007).

1.3.2. Factors influencing outbreaks of parasitic diseases in sea-cages

Sea-cage cultured fish species are affected by both endoparasites (e.g. microsporidians, and myxozoans) and ectoparasites (e.g. protozoans, monogeneans, and crustaceans); however the latter are the most prevalent (Nowak, 2007). Sometimes free-living organisms (e.g. protistans *N. perurans* and *Uronema nigricans*) that are not found to infect a certain fish species in the wild might behave as opportunistic parasites and parasitise the same farmed species (Nowak, 2007). So far, the low genetic diversity (Ebert, 1998), continuous input of naive individuals and high density of host populations seem to be the key for the success of parasites in sea-cage culture (Nowak, 2007). In fact, parasites are able to adapt to specific genotypes and increase their virulence in homogeneous host populations that are the result of clonal or inbreeding programs (Ebert, 1998). Thus, maintaining heterogeneity within a farmed fish population is critical to containing outbreaks of parasitic diseases (Lively et al., 1990). Genetic variation in a farmed fish population is usually maintained through good hatchery management, although cases of genetic diversity reduction have been previously

reported in farmed populations of salmonids (Kim et al., 2004; Norris et al., 1999), turbot (*Scophthalmus maximus*) (see Coughlan et al., 1998) and sea-bass (*Dicentrarchus labrax*) (see Bahri-Sfar et al., 2005).

Another key factor influencing the success of parasitic infections in sea-caged fish is the mechanism of transmission between hosts. In fact, the major part of parasitic diseases use horizontal transmission which allows them not to rely on the host survival or reproduction (Galvani, 2003). Furthermore, it has been shown that dead fish in sea-cages might represent a reservoir of infection as they can be colonised post-mortem (Douglas-Helders et al., 2000).

Homogeneity in sea-cage fish populations is another relevant factor that has contributed to determining the biodiversity of parasitic organisms that can cause disease in the cage environment which is quite low compared to the wide range of different parasites infecting the same fish species in the wild. For example, in Australia only two monogenean ectoparasites are known to represent a serious concern for the cage-culture of yellowtail kingfish (*Seriola lalandi*) (see Chambers and Emst, 2005; Mansell et al., 2005; Tubbs et al., 2005) whereas in the wild, 17 ectoparasites and 41 endoparasites have been found to infect *Seriola* spp. (Hutson et al., 2007). Generally, the parasites that cause infection outbreaks in mariculture are mostly ectoparasites with a direct life-cycle and short generation time (Nowak, 2007). However, there are also exceptions (e.g. blood fluke species from the genus *Cardicola*). One main explanation to this predominance of ectoparasitism is related to the fact that in mariculture manufactured feeds predominate which prevents the transmission of parasites (e.g. some metazoan species) that enter the host through trophic activities (Nowak, 2007). Moreover, this higher prevalence of ectoparasitism and low diversity at the species level in cage-culture has been also associated with the eutrophication and/or pollution of seawater (Kesting and Zander, 2000; Lafferty, 1997; MacKenzie, 1999).

To prevent and/or control the outbreaks of parasitic diseases in mariculture research is focused mainly on improving or developing selective breeding programs, vaccines, mitigation treatments, diets, immunostimulants and the general health management of fish stocks.

1.4. Atlantic salmon – *Salmo salar* Linnaeus, 1758

1.4.1. Life history

The Atlantic salmon, *S. salar*, belongs to the salmonid subfamily Salmoninae that includes about 30 fish species in seven genera (Nelson, 2006). Wild Atlantic salmon distribution covers the northern European and American regions of the Atlantic ocean (FAO). In contrast, the Atlantic salmon in aquaculture systems are found worldwide including the Pacific waters such as British Columbia in Canada and the Southern hemisphere, mainly Chile and Tasmania (Gross, 1998). *S. salar* is mostly an anadromous fish species; this means that part of its life cycle (**Fig. 1.3.**), the adult stage, is spent in the marine environment and that the adults migrate to the freshwater environment only to spawn (Jobling, 2010). During the marine phase of their life the adults prey on a wide variety of organisms like crustaceans, amphipods, decapods and other fish species such as sand lance, smelt, alewives, herring, capelin, mackerel juveniles and cod juveniles (Caspers, 1976). In freshwater systems, the early life stages have a different diet preying mainly on aquatic insects (Caspers, 1976). The Atlantic salmon is also an iteroparous fish species and as such it may spawn several times in its life (Gross, 1998). One of the most interesting aspects of the life cycle of this salmonid species is the transformation from parr to smolt life stage which involves changes in the osmoregulatory system of the fish (Jobling, 2010). In fact, the transition from a freshwater to a marine environment requires changes in the ionic regulatory mechanisms of the fish, particularly in the gills (Jobling, 2010).

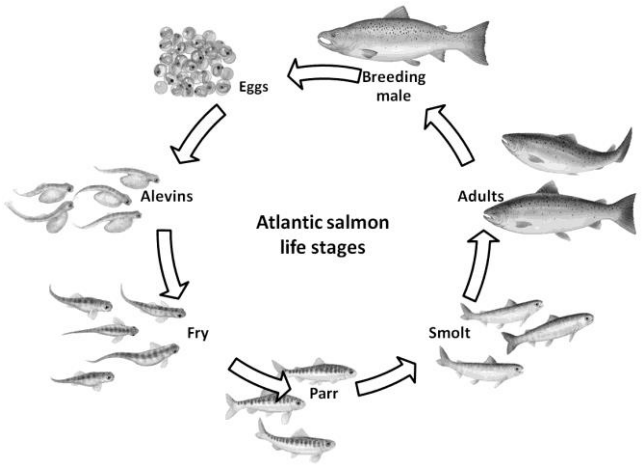

Fig. 1.3. Atlantic salmon life cycle stages. Modified from (MSA).

1.4.2. Farming history

Atlantic salmon farming became popular at the end of the 20th century and with more than 1 million tonnes annually produced, is nowadays an established industry worldwide (FAO; Jobling, 2010). Norway was the first country to farm the Atlantic salmon in sea net-cages in 1960, rapidly followed by Scotland and more recently Ireland, the Faroe Islands, Canada, USA, Chile and Australia (Tasmania) (FAO). The farming of Atlantic salmon takes place also in France and Spain, although to a lesser extent (FAO). The rearing techniques are relatively similar for all countries where this salmonid species is farmed, consisting of egg incubation, alevin and fry rearing, early smolt production in freshwater systems and marine grow-out stage (Jobling, 2010). The latter stage ends when the individuals reach 3-7 kg, which is considered the market-size (Jobling, 2010).

1.4.2.1. Atlantic salmon farming in Tasmania

Mariculture, although regionally based, is the fastest growing primary industry in Australia and since 1990 increased in value by an average of 13% per year (AFFA, 2002). Many marine organisms are cultured in Australia (e.g. pearls, oysters, prawns and Southern bluefin tuna) but more than one half of the total value production (55%) is represented by salmonids, mainly Atlantic salmon (Stephan and Hobsbawn, 2015). Atlantic salmon is not a native species to Australia and initially was introduced from Canada to New South Wales, Australia in the mid-1960s (ABARES, 2003). Tasmania acquired eggs from the broodstock of Atlantic salmon held in New South Wales and started its commercial production in the mid-1980s (ABARES, 2003). Nowadays, in Australia, the Atlantic salmon production is located almost entirely in Tasmania, with the exception of regional farming in South Australia off Cape Jaffa and in the north east of Victoria (freshwater systems) (ABARES, 2003). In the period 2013-14 the Tasmanian Atlantic salmon production was around 42,000 tonnes (AUD 531.3 million) becoming the largest and most valuable mariculture production in Australia (Stephan and Hobsbawn, 2015). The support of research, mainly focused on feeding techniques and

disease management has been important for the successful growth of the Tasmanian salmonid industry (Stephan and Hobsbawn, 2015).

1.5. Amoebic gill disease – with a focus on Atlantic salmon in Tasmania

Amoebic gill disease (AGD) is a branchial disease associated with colonisation of the gill epithelium by *N. perurans* (see Young et al., 2007). Although a marine free-living protozoa, *N. perurans* is also capable of invading the fish gills and acting as a pathogen (Nowak, 2012). Previous studies showed that high salinity is one of the major environmental risk factors for AGD outbreaks (Adams and Nowak, 2003; Bustos et al., 2011; Clark and Nowak, 1999; Munday et al., 1990; Nowak, 2001). This ectoparasitic amoeba is one of the major threats to the Atlantic salmon industry in Tasmania (Munday et al., 2001; Oldham et al., 2016). AGD outbreaks often result in a consistent reduction of profits mostly due to the use of freshwater bathing of the AGD-affected fish as a disease mitigation strategy (Munday et al., 2001). In fact, if the AGD-affected fish are not treated with a freshwater bath, the mortality of the affected individuals can be over 50% (Munday et al., 1990). Furthermore, following the industrial freshwater bath, reinfection events are common and usually in summer temperatures, it takes only 2 weeks before the disease spreads again (Adams and Nowak, 2004).

1.5.1. Geographical distribution of AGD and affected fish species

The first reports of AGD date back to more than 20 years ago and were observed in farmed Atlantic salmon and rainbow trout (in Tasmania (Australia) (Munday, 1986) and in farmed coho salmon (*Oncorhynchus kisutch*) in the State of Washington (USA) (Kent et al., 1988). To date, AGD has been confirmed to affect farmed Atlantic salmon in several countries such as Australia (Munday et al., 1990), Chile (Bustos et al., 2011), France (Findlay and Munday, 1998), Ireland (Palmer et al., 1997; Rodger and McArdle, 1996), Spain (Rodger and McArdle, 1996), United States of America (Douglas-Helders et al., 2001), Norway (Steinum et al., 2008), United Kingdom (Steinum et al., 2008), South Africa (Mouton et al., 2014) and recently Canada (Oldham et al., 2016). Furthermore,

this disease is known to represent a threat not only for salmonids but also for other farmed marine fish species in different geographic locations. In the Mediterranean sea, AGD was reported for turbot (*Scophthalmus maximus*) (see Dykovàa et al., 1995), seabass (*Dicentrarchus labrax*) (see Dykovàa et al., 2000), sharpsnout seabream (*Diplodus puntazzo*) (see Dykovàa et al., 2000), and seabream (*Sparus aurata*) (see Athanassopoulou et al., 2002). In Asia, AGD was found to affect ayu (*Plecoclossus altivelis*) in Japan (Crosbie et al., 2010) and olive flounder (*Paralichthys olivaceus*) in Korea (Kim et al., 2005b). In Norway, a cultured cleaner fish, ballan wrasse (*Labrus bergylta*), used in Atlantic salmon farms has also shown signs of AGD (Karlsbakk et al., 2013). An opportunistic sampling highlighted the presence of *N. perurans* in the gills of the blue warehou (*Seriolella brama*) collected from an Atlantic salmon farm cage in Tasmania (Adams et al., 2008). Recently, during a large-scale marine survey in Scotland, real-time quantitative polymerase chain reaction (qPCR) showed that a wild individual of horse mackerel, *Trachurus trachurus*, was positive to *N. perurans* (see Stagg et al., 2015). Therefore, according to the research carried out until now wild fish are not a significant reservoir of *N. perurans* (see Oldham et al., 2016).

1.5.2. Diagnosis and pathogenesis

Typical signs displayed by AGD-affected fish are macroscopic white patches (AGD-lesions) on the gills associated with excessive mucus production (Munday et al., 1990). As industry practice, Tasmanian salmon farmers use the presence of these lesions on the gills to diagnose AGD and as a gross measurement of AGD severity (Adams et al., 2004). Moreover, the gill score practice allows the farmers to determine when a freshwater bathed is needed (Adams et al., 2004) and also to evaluate the AGD severity after the administration of different experimental treatments (Florent et al., 2007; Florent et al., 2009).

AGD-lesions are associated with epithelium hyperplasia (Adams et al., 2004; Dykovàa et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Zilberg et al., 2001), that in the most severe cases can result in a lamellar fusion affecting the whole filaments (Adams et al.,

2004). Usually, the amoebae are visualised in the gill histological sections of AGD-affected fish (Adams and Nowak, 2003). However, to confirm the pathogen identity as *N. perurans*, either PCR or *in situ* hybridization is needed (Young et al., 2007; Young et al., 2008b; Young et al., 2008c). In fact, there are no morphological differences that can be used to distinguish between different species belonging to the genus *Neoparamoeba* (see Dykovàa et al., 2005; Young et al., 2007).

1.5.3. Host immune gene expression

In the last decade there was an increase in studies using either qPCR or cDNA microarrays to investigate the salmonid immune response against *N. perurans* at the transcriptional level (Bridle et al., 2006a; Bridle et al., 2006b; Loo et al., 2012; Morrison et al., 2006a; Morrison et al., 2006b; Morrison et al., 2007; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a). The main reason for this increase is that the sequences of genes related to the inflammatory and immune response in salmonids have become more available for gene expression research (Davidson et al., 2010).

The previous studies on the host inflammatory and immune gene expression following challenge with *N. perurans* focused mainly on the differences between AGD-lesion and control (non-lesion) area in AGD-affected fish and also between AGD-affected and unaffected fish (Bridle et al., 2006a; Bridle et al., 2006b; Loo et al., 2012; Morrison et al., 2006a; Morrison et al., 2006b; Morrison et al., 2007; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a). In particular, in AGD-affected Atlantic salmon, the gene expression of the pro-inflammatory cytokine interleukin (IL)-1β was significantly increased throughout the disease progression (Bridle et al., 2006a; Loo et al., 2012; Morrison et al., 2007; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a). Furthermore, the cytokine tumour necrosis factor (TNF)-α and the chemokine IL8 transcripts were also increased respectively during early and late infection in AGD-affected Atlantic salmon (Morrison et al., 2012; Young et al., 2008a). However, the up-regulation of a few cytokines was not enough to suggest the involvement of an inflammatory cytokine cascade during AGD (Nowak et al., 2014). In general, a

coordinated down-regulation of immune-related genes was reported during late stages of the disease (Young et al., 2008a). Most notably, this decrease of the immune gene expression involved different genes related to the major histocompatibility complex (MHC) class I and MHC class II antigen processing and presentation pathways (Young et al., 2008a). Microarray studies investigated the early stages of infection and reported changes in various immune transcripts such as CC chemokine, C type lectin and T cell receptor (TCR)-α (Morrison et al., 2006a).

1.5.4. Protective strategies

To date, several experimental trials to boost the fish immune system and reduce the risk of infection were attempted. A variety of immunostimulant treatments and experimental vaccines were tested showing no or little effect on the survival of AGD-affected fish (Nowak et al., 2014). A recent attempt to develop a vaccine against *N. perurans* used a recombinant protein r22C03 which simulated an amoebic attachment factor (Valdenegro-Vega et al., 2015a). However no difference in survival was observed between the vaccinated and the control group (Valdenegro-Vega et al., 2015a). More promising was the injection of CpGs (Bridle et al., 2003) which showed a 38% increase in protection against the pathogen, and two experimental diets containing immunostimulants (Dick, 2012) which increased fish survival by 27%.

1.5.5. Knowledge gaps

There are still many knowledge gaps on the host immune response to *N. perurans*. To increase our knowledge of the fish immune response is critical to develop future strategies aimed at boosting the host immune system (e.g. immunoprophylaxis) and providing protection against AGD. To date, several studies have been carried out on AGD mainly focusing on late disease stages; however, none of them showed if *N. perurans*, the etiological agent of the disease, elicits either an innate or adaptive immune response in the host. In particular, previous works showed that the pro-inflammatory cytokines IL-1β (12, 14, 25, 36, 38 days post-exposure) and TNF-α (12 days post-exposure) were significantly induced in the AGD-lesions with gross lesions detected. Furthermore, other studies

showed that during early infection (0, 2, 5 days post-exposure) eight immune-related genes were up-regulated and only two down-regulated in AGD-affected gills with no gross lesions observed. The same authors (Morrison et al., 2006a; Morrison et al., 2006b) reported that at 8 days post-exposure five immune-related genes were down-regulated and three up-regulated in AGD-affected gills with gross lesions. However, more recent research (Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a) suggested a coordinated decrease of immune-related genes in the host gills following infection with *N. perurans* during late stages (19, 25, 36 days post-exposure) of AGD in the presence of gross lesions and a high percentage of affected filaments. Although reinfection with *N. perurans* following freshwater bathing is very common in Atlantic salmon farming, there is little knowledge about the inflammatory and immune processes in the host gills following multiple exposures to the parasite. Overall, clarification is needed on the inflammatory and immune processes in the AGD-affected gills, particularly on the earlier disease stages, following a single and multiple exposures to *N. perurans*.

1.6. Pacific bluefin tuna – *Thunnus orientalis* (Temminck e Schlegel, 1844)

1.6.1. Life history

The Pacific bluefin tuna (PBT) is one of the most commercially valuable fish species and belongs to the Scombridae family which includes other species relevant to the fishery and aquaculture industry such as the Atlantic or Northern bluefin tuna (*Thunnus thynnus*), the Southern bluefin tuna (*Thunnus maccoyii*) and more recently the yellowfin tuna (*Thunnus albacores*) (see Ottolenghi, 2008). Bluefin tuna are very large (maximum length 4.6 m and weight 684 kg) and long-lived (lifespan 35-50 years) fish species and have a unique physiology which includes a specialised continuous swimming, high cardiac output and partial endothermy that allows them to exploit an expanded thermal niche (from temperate to subtropical waters) compared to other fish species (Graham and Dickson, 2004). Bluefins make long annual migrations to feed mainly on other smaller

fish species, cephalopods, and crustaceans (Graham and Dickson, 2004). Furthermore, bluefin tuna make also feeding vertical migrations since they are able to dive at extremely deep depth (maximum depth 914 m) (Graham and Dickson, 2004). The PBT occurs in tropical to temperate waters of the Pacific Ocean preferring the North Pacific with occasional presence in the Southern Hemisphere (**Fig. 1.4.**) (ISC). PBT are iteroparous fish, which means they spawn several times in their lifetime (ISC). The spawning area is located in the North Western Pacific Ocean and the spawning season usually occurs between May and June and from late June to August (**Fig. 1.4.**) (ISC).

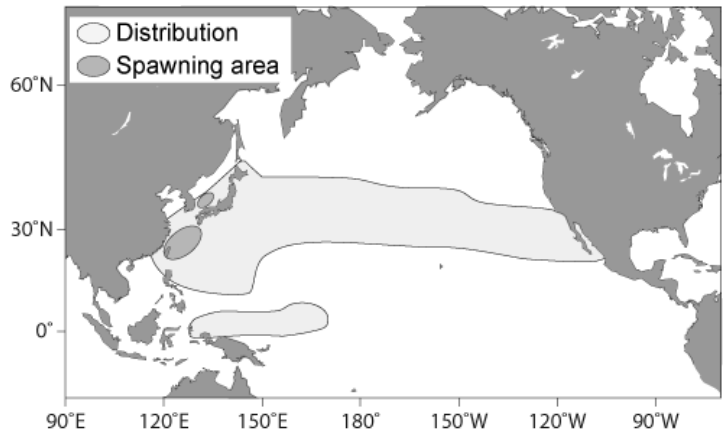

Fig. 1.4. Geographical distribution and spawning area of *T. orientalis* (ISC).

1.6.2. Stock status

T. orientalis represents an international fishery resource and as such its catch is strictly controlled by two international bodies: the Western and Central Pacific Fisheries Commission (WCPFC) and the Inter-American Tropical Tuna Commission (IATTC). The most recent PBT stock assessment conducted by the International Scientific Committee for Tuna and Tuna-like Species in the North Pacific Ocean (ISC) showed that the spawning stock biomass is near to its historically lowest level (ISC, 2014). Furthermore, in 2014 the International Union for Conservation of Nature (IUCN) updated the status of *T. orientalis* from "Least Concern" to "Vulnerable" in its red list of threatened species (IUCN, 2014). More precisely, on the red list, the label of "Vulnerable" for an animal species means that it is considered to be facing a high risk of extinction in the wild (IUCN).

1.6.3. Farming history with a focus on Japan

Worldwide the bluefin tuna aquaculture is based on the fattening of young fish captured from the wild and ranched until the market size (Benetti et al., 2016). In Japan, the PBT larviculture and broodstock management date back to 1970 (Masuma et al., 2011), however, the farming of this fish species at a commercial scale started only during the early 1990s (Tada, 2010). To date the Japanese PBT farmed production accounts for around 9,000 metric tonnes/annum (Tveteras, 2015) of which around 200 metric tonnes were obtained from hatchery-reared juveniles (Benetti et al., 2016). The greatest achievement in the bluefin tuna farming history was accomplished in 2002 by Kinki University, in Japan, that after 32 years of research, managed to fully close the PBT life-cycle (**Fig. 1.5.**) in captivity under controlled conditions (Kumai and Miyashita, 2003; Sawada et al., 2005). This success was also attained thanks to the collaboration of the Japanese federal government (Fisheries Research Agency), prefectural governments, and private companies that promoted research aimed to improve the PBT rearing technology (Benetti et al., 2016). However, there are still issues related to the PBT larval rearing that need to be addressed to increase the juvenile production and consequently reduce the input of wild fish. Some of the most relevant concerns are the so-called "sinking syndrome" which affect larvae during the first 8 days of life (Ishibashi, 2010), formulation of an appropriate larval and early juveniles diet, cannibalism, collisions to the tank walls, transfer from the

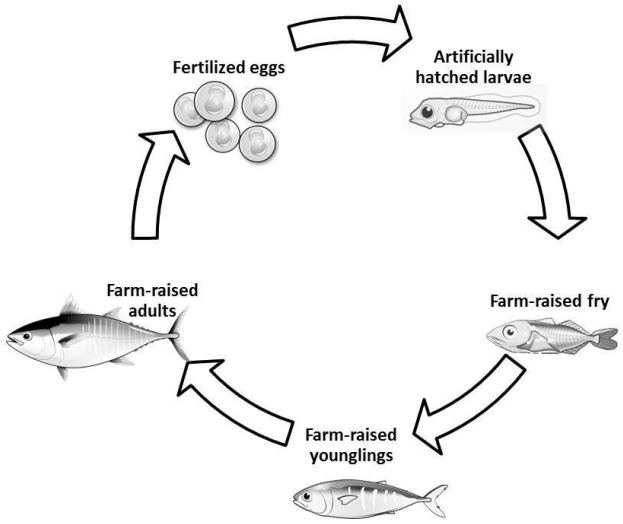

Fig. 1.5. Complete PBT life cycle in captivity in Japan. Images courtesy of Dr Shirakashi (Aquaculture Research Institute, Kindai University, Nishimuro, Wakayama, Japan).

land based facilities to the sea-net cages, skeletal anomalies, and infectious diseases (Masuma et al., 2011).

Mexico, with 4,500 metric tonnes/annum, is the second highest producing country of *T. orientalis* and, due to the PBT migration pattern across the Pacific, shares the same stock with Japan (Buentello et al., 2016). However, as for the Mediterranean countries and Australia, Mexico uses a bluefin tuna ranched-based aquaculture, and there have been no attempts to start hatchery production (Buentello et al., 2016). Even if negligible from a production point, Korea is following the Japanese farming style and has started a PBT hatchery production which allows the country to produce their own farmed juveniles (Benetti et al., 2016).

1.6.4. Infectious diseases in farmed and ranched bluefin tuna

Bluefin tuna, both farmed and ranched, are confined in sea net-cages submerged in the marine environment and, as such represent an easy target for parasites. In fact, the net-cage is not a controlled system like a tank and offers an easy entry for pathogens (Nowak, 2007). Furthermore, the cage environment may facilitate free living waterborne organisms to turn into parasites increasing the number of different parasitic species infecting the bluefin (Nowak, 2007). There are no records for viral diseases in ranched *T. thynnus* and *T. maccoyii*. Nevertheless, two viral infections, the red sea bream iridovirus (RSIV) (Kawakami and Nakajima, 2002; Sawada et al., 2005) and the viral nervous necrosis (VNN) caused by the piscine nodavirus (*Betanodavirus*, *Nodaviridae*) (Nishioka et al., 2010), have been reported to cause significant mortality in farmed larvae and juveniles of *T. orientalis*. Bacterial disease outbreaks are mostly opportunistic in all the three bluefin tuna species and have been recorded associated with a low-quality diet (ABT) (Mladineo et al., 2006), parasitic lesions (SBT) (Munday et al., 2003), and post-transfer stress (PBT) (Balli et al., 2016). Protozoan infections were not associated with pathological changes in ABT (Mladineo, 2006), whereas in PBT embryos and yolk-sac larvae, an endoparasitic protozoan belonging to the genus *Ichthyodinium* sp. caused severe losses in the juvenile production (Ishimaru et al., 2012). Moreover, in SBT the

scuticociliate *Uronema nigricans* has been related to "swimmer syndrome", that is a severe locomotory dysfunction (Balli et al., 2016). However, in the last decade, the improved husbandry practice in SBT ranching has significantly reduced the risk of infection to a few reported cases (Balli et al., 2016). Several different other parasites such as microsporidians, kudoids, dydimozoans, and crustaceans, have been found in bluefin tuna, although with a limited etiological significance and no major pathology (Balli et al., 2016).

1.7. *Cardicola* spp. infection in bluefin tuna

1.7.1. Cardicola spp. geographical distribution

Among all the parasitic species that infect bluefin tuna the blood flukes ascribed to the genus *Cardicola* represent the major concern for the industry, particularly for the farmed juvenile PBT in Japan (Ogawa, 2015) and for the ranched adults of SBT in Australia (Aiken et al., 2009; Cribb et al., 2011; Polinski et al., 2013c). To date, three *Cardicola* spp., *C. orientalis*, *C. opisthorchis* and *C. forsteri* are known to infect farmed *T. orientalis* (see Ogawa et al., 2010; Ogawa et al., 2011; Shirakashi et al., 2016). The latter species, *C. forsteri*, has been very recently reported in Japan in PBT (Shirakashi et al., 2016) and was previously described in co-infection with *C. opisthorchis* and *C. orientalis* in the Mediterranean ABT (Palacios-Abella et al., 2015) and co-infecting the Australian SBT with *C. orientalis* (see Aiken et al., 2009; (2011); Polinski et al., 2013c; Shirakashi et al., 2013). After less than a decade of research on *Cardicola* spp. infections and their fish hosts is emerging that these parasites are not as species specific as was initially believed and that they seem to have a similar geographic distribution.

1.7.2. Cardicola spp. life cycle

The fish blood flukes like *Cardicola* spp. are trematodes belonging to the Aporocotylidae family and typically parasitise the vascular system of the host (Smith, 2002). The life cycle (**Fig. 1.6.**) of these parasites includes asexual reproduction in an invertebrate intermediate host and sexual

reproduction in a vertebrate definitive host (Cribb et al., 2011), the bluefin tuna in the case of *C. forsteri*, *C. orientalis* and *C. opisthorchis*.

All the described marine blood flukes with known life cycles use a polychaete as intermediate host, although larval stages of unknown aporocotylids species have also been reported from bivalves (Cribb et al., 2011; Orelis-Ribeiro et al., 2014). Recently it has been revealed that the *Cardicola* spp. infecting the Japanese PBT all use terebellid polychaetes as the intermediate host (Shirakashi et al., 2016). In particular, Sugihara et al., (2014) demonstrated that *C. opisthorchis* larval stages (sporocysts and cercariae) use as intermediate host a terebellid polychaete belonging to the genus *Terebella*. In a recent work, Shirakashi et al., (2016) found that developing sporocysts and mature cercariae of *C. orientalis* use as intermediate host the polychaete *Nicolea gracilibranchis*. Furthermore, the same authors reported a polychaete of the genus *Amphitrite* hosting *C. forsteri* cercariae (Shirakashi et al., 2016), while in Australian SBT the intermediate host has been identified with the terebellid *Longicarpus modestus* (see Cribb et al., 2011). Moreover, the above-mentioned study showed that these polychaetes hosting *Cardicola* spp. life stages prefer cage floats and ropes to frames and bottom substrate (Shirakashi et al., 2016).

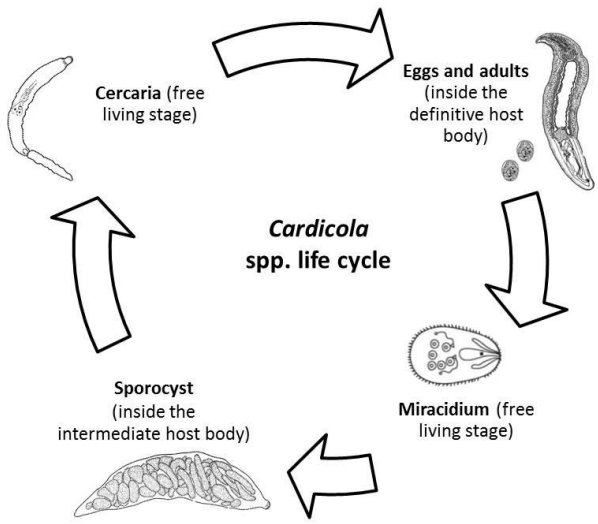

Fig. 1.6. *Cardicola* spp. life cycle in bluefin tuna. Images from (Cribb et al., 2011; David Ian Gibson, 2002; Shirakashi et al., 2013).

In general, following asexual reproduction and the release of cercariae from the intermediate host, the cercariae as a *Cardicola* spp. free living stage swim until they find a definitive host to penetrate and infect (Cribb et al., 2011). Once the adult blood flukes have settled in the fish, they lay the eggs that are carried to the gills where they lodge (Cribb et al., 2011). After the hatching, the miracidia are released and they exit the host looking for the intermediate host (Cribb et al., 2011). From a previous study on *Cardicola* spp. distribution in PBT, we know that *C. orientalis* eggs and adults mainly infect the gills whereas *C. opisthorchis* eggs are found in the gills and the adults in the heart (Shirakashi et al., 2012b). Furthermore, *C. orientalis* and *C. opisthorchis* have a different egg shape and site of accumulation in the gills, as the former species lays smaller oval-shaped eggs in the gill lamellae whereas the latter larger crescent-shaped eggs in the gill filament arteries (Shirakashi et al., 2012b). Although *C. forsteri* has been only recently detected in PBT, the distribution of this species in the host is known from previous works on SBT in Australia (Cribb et al., 2011). These works showed that in SBT the adults of *C. forsteri* are found in the heart and the eggs in both heart and gills (Colquitt et al., 2001; Cribb et al., 2011). Nevertheless, more research needs to be done to find out if *C. forsteri* has a similar distribution in PBT.

1.7.3. Diagnosis, detection, pathology, and treatment

In both SBT and PBT industry *Cardicola* spp. infections have been associated with mortality of untreated fish (Cribb et al., 2011; Polinski et al., 2013c; Shirakashi et al., 2012b). Clinical signs associated with *Cardicola* spp. infections are increased mucus production on the gills, respiratory distress and lethargy (Munday et al., 2003).

The diagnosis of *Cardicola* spp. infection is quite invasive since it relies mostly on the heart flushing and then checking the flushes for the adults and gill microscopy (either fresh preparations or histology) to visualise the eggs (Munday et al., 2003; Shirakashi et al., 2012b). Recently, a sensitive and qPCR technique was developed for the detection of *Cardicola* spp. DNA in SBT (Polinski et al., 2013c) and PBT (Polinski et al., 2014b) gills, heart and serum. This molecular procedure exceeds all

the previous detection methods used, since it not only allows researchers to be more precise at recognising the onset of the infection, but also it is extremely useful for mapping the *Cardicola* spp. DNA in the host (Polinski et al., 2013c; Polinski et al., 2014b). Nevertheless, this qPCR method has a few relevant limitations, as it is not able to distinguish between different *Cardicola* spp. life stages in the host and if the parasites detected are alive or dead (Polinski et al., 2014b).

The pathology in both bluefin species is associated with the accumulation of the parasite's eggs in the gills which can cause a severe host inflammatory response (branchitis) and affect the blood flow eventually leading the fish to suffocate to death (Colquitt et al., 2001; Munday et al., 2003; Shirakashi et al., 2012b). Furthermore, histopathological examinations showed that *Cardicola* spp. eggs physically damage the gills causing a widespread fusion and clubbing of the lamellae (Shirakashi et al., 2012b). In PBT it has been suggested that *C. orientalis*, more than *C. opisthorchis*, represents a major risk due to the fact that its eggs accumulate in the gill capillaries and its adults reside in the branchial arteries which may clog the gill arteries reducing the blood flow (Shirakashi et al., 2012b). PBT juveniles up to 1-year-old are the most vulnerable to disease (Shirakashi et al., 2012b).

The only effective treatment used in the PBT and SBT industry against *Cardicola* spp. is the administration of an antihelminthic drug, praziquantel (PZQ), which effectively eradicates the parasite adult life stages (Shirakashi et al., 2012a). Usually, reinfection happens following the first PZQ treatment, thus treatment at each *Cardicola* spp. infection outbreak is necessary to keep the fish free from the parasites (Balli et al., 2016; Hardy-Smith et al., 2012; Shirakashi et al., 2012a). The effectiveness of this drug has been attributed to an increased parasite antigen presentation activity promoted by T cells and B cells following PZQ treatment (Brindley and Sher, 1987; Harnett and Kusel, 1986; Sabah et al., 1985). Furthermore, a recent *in vitro* study demonstrated that PZQ has an immunomodulatory ability in fish (Polinski et al., 2014a).

1.7.4. Host immune gene expression

Although the *Cardicola* spp. infections are considered the major threat to the bluefin tuna industry, very little is known about the host immune response to these parasites. Furthermore, the fish immune response to different blood fluke species other than *Cardicola* spp. is also largely unknown. There is only one previous study that investigated the PBT immune response at the mRNA level during co-infection with *C. orientalis* and *C. opisthorchis* (Polinski et al., 2014b). Transcription of immune-related genes IgM, MHC-II, TCR-β and IL8, was significantly increased in the heart of the infected fish and IgM was also significantly increased in the gill of the infected fish relative to uninfected individuals (Polinski et al., 2014b). Furthermore, the same authors observed that there was a strong positive correlation between the relative quantity of IgM transcript and the relative abundance of *C. orientalis* but not *C. opisthorchis* DNA in the host gills, suggesting that the parasites, particularly *C. orientalis*, triggered a cellular immune response in the host (Polinski et al., 2014b). Nevertheless, in the same work, no change in the expression of two cytokine transcripts, TNF2 and IL-1β, involved in the host inflammatory response could be detected in PBT gills and heart during *Cardicola* spp. infection (Polinski et al., 2014b). However, another study reported evidence of an inflammatory process in wild PBT gills and skin infected by dydimozoid *Didymocystis wedli* (see Mladineo and Block, 2010). This observation of inflammation was supported by the up-regulation of the two cytokine transcripts IL-1β and TNF-α at the infection site (Mladineo and Block, 2010). Moreover, serological evidence showed that juvenile SBT infected with *C. forsteri* are able to develop an antibody response which coincides with the appearance of the parasite eggs (Aiken et al., 2008; Kirchhoff et al., 2012).

A recent work on the *in vitro* effects of PZQ on the fish immune system provided evidence that this drug, independently of parasitic antigen, modulated the transcriptional response of inflammatory and immune-related genes (Polinski et al., 2014a). In particular, the SBT blood cell culture stimulated with PZQ showed an up-regulation of the inflammatory cytokines IL-1β, TNF2, of the chemokine IL8 and of the immune-related genes TCR, IgM, and MHC-I, whereas in the SBT,

intestinal explants stimulated with PZQ were observed to increase IL8, TCR and IgM (Polinski et al., 2014a). The authors suggested that the use of PZQ in fish parasitosis enhanced the T-cell, B-cell, and inflammatory associated signalling response in the host (Polinski et al., 2014a).

1.7.5. Knowledge gaps

At present, very little is known on host immunity against *Cardicola* spp. infection, in particular there is no information available on the PBT early immune response. The transfer of PBT early life-stages to sea-cages is critical as the young fish are exposed for the first time to the marine environment and to possible pathogens such as *Cardicola* spp. Therefore, understanding the PBT early immune response against the parasites could be of utmost importance to developing future immunisation strategies to protect the young fish against *Cardicola* spp. infection. At the moment, the only treatment to mitigate the infection by *Cardicola* spp. is PZQ, an anthelminthic drug which has been associated with immunostimulant effects. Nevertheless, there is little information available on the ability of PZQ to boost the fish immune system or immune response during early rearing.

1.8. Objectives of this thesis

Cardicola spp. infection and AGD, caused by *N. perurans*, represent a great concern for the sea-cage culture of, respectively, PBT in Japan and the Atlantic salmon in Australia (Tasmania). Both *Cardicola* spp. and *N. perurans* can cause severe pathology to the gills of infected fish which in the most critical cases may result in fish death. The only effective treatments used to mitigate *Cardicola* spp. is the oral administration of PZQ and *N. perurans* infections are treated using a freshwater bath of the affected fish. Immunoprophylaxis may represent a future valid alternative to protect the fish against *Cardicola* spp. infection and AGD following the transfer of the fish from land-based facilities to the sea-net cages. However, little is known about the inflammatory and immune processes in the host gills which are the main infection site. Therefore, research in this thesis has been focused toward addressing the following objectives:

- Investigate the host inflammatory and immune response in the AGD-affected gills of Atlantic salmon following a single early exposure, a single long-term exposure and multiple exposures to *N. perurans* by using a qPCR.
- Describe the inflammatory and immune response in the gills of early life stages of PBT infected by *C. orientalis* and *C. opisthorchis* by using a qPCR and histopathology.
- Describe the *Cardicola* spp. infection timeline following the transfer of early life stages of PBT to sea-cages and determine the relative prevalence of *C. orientalis* and *C. opisthorchis* DNA in PBT gills and heart by using qPCR.
- Determine the effects of praziquantel treatment on the PBT immune response and *Cardicola* spp. prevalence.

CHAPTER 2

EVIDENCE OF IMMUNE AND INFLAMMATORY PROCESSES IN THE GILLS OF AGD-AFFECTED ATLANTIC SALMON, *SALMO SALAR* L.

Pennacchi, Y., Leef, M.J., Crosbie, P.B.B., Nowak, B.F., Bridle, A.R. (2014). Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. Fish and Shellfish Immunology, 36: 563-570.

2.1. Abstract

Amoebic gill disease (AGD) is a disease caused by the ectoparasite *N. perurans* which affects several cultured marine fish worldwide. The characterisation of pro-inflammatory and immune related genes at the mRNA level in AGD-affected Atlantic salmon gills was performed at 10 days post-inoculation using 2D qPCR, a method of mapping transcriptional responses in tissues. The genes of interest were IL-1β, TNF-α, TCR- α chain, CD8, CD4, MHC-IIα, MHC-I, IgM and IgT. A significant increase in expression of the mRNA of all the genes was observed in the gills of AGD-affected fish. Contrary to previous studies, our data suggest that the parasite, *N. perurans*, elicits a classical inflammatory response in the gills of AGD-affected fish and indicates that the mRNA expression of immune genes within gill lesions misrepresents the cellular immune response in the gills during AGD.

2.2. Introduction

Amoebic gill disease (AGD) is an ectoparasitic infection of the gills of an increasing number of marine fish species farmed in sea net cages (Dykovàa et al., 1995; Kent et al., 1988; Munday, 1986). This disease is caused by *N. perurans* (see Young et al., 2007) an amphizoic amoeba which is the confirmed aetiological agent of AGD in cases reported from Australia, Ireland, Japan, New Zealand, Norway, USA, Scotland, Spain, France, Chile and most recently South Africa (Bustos et al., 2011; Crosbie et al., 2010; Crosbie et al., 2012; Mouton et al., 2014; Nylund et al., 2008; Steinum et al., 2008; Young et al., 2008c). AGD is the most significant health problem affecting the production of Atlantic salmon, *S. salar* L. in Australia and causes mortalities of over 50% if fish are not treated (Munday et al., 1990). Currently the only economically viable treatment consists of freshwater bathing of afflicted fish for 2-4 hours (Parsons et al., 2001) which represents a substantial economic disadvantage for the industry contributing up to 20% of total production costs (Munday et al., 2001). Untreated fish display macroscopic multifocal white patches on the gills, leading to excess mucus

production and respiratory distress (Munday et al., 1990). Histologically, AGD is associated with epithelial hyperplasia (Roubal et al., 1989). Despite the seriousness of this disease and its increasing impact on the salmonid industries in the UK, Ireland, Norway and Chile, little is known about the involvement of the host immune response to infection with *N. perurans* and AGD. Previous studies showed no evidence that AGD-affected fish have the capacity to develop innate (Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2007; Young et al., 2008a) or acquired immunity (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross et al., 2004a; Gross et al., 2006; Morrison et al., 2006a; Vincent et al., 2006; Young et al., 2008a) to the parasite. Early studies of the transcriptional response to AGD have provided a limited understanding about the Atlantic salmon immune response to *N. perurans*. Most notable from these studies is the fact that *N. perurans* was shown to induce the mRNA expression of IL-1β and TNF-α (Bridle et al., 2006a; Loo et al., 2012; Morrison et al., 2007; Morrison et al., 2012; Young et al., 2008a) specifically into the lesions of AGD-affected Atlantic salmon gills (Morrison et al., 2007; Young et al., 2008a). Although these studies provided evidence of an increased expression of two important inflammatory mediators subsequent studies contradicted the involvement of an inflammatory process and even suggested the down-regulation of important immune and inflammatory genes (Loo et al., 2012; Morrison et al., 2006a; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a).

Based on our hypothesis that immune signalling in AGD-affected gills is highly dependent on the ratio of normal to hyperplastic gill tissues the purpose of this study was to investigate the host immune response in AGD-affected Atlantic salmon gills using a 2D quantitative RT-PCR, which is a method of mapping transcriptional responses in tissues. In particular the study focused on the transcriptional expression of the following inflammatory and immune related genes: interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), T-cell receptor alpha chain (TCR-α chain), cluster of differentiation 8 alpha (CD8-α), cluster of differentiation 4 alpha (CD4-α), major histocompatibility complex I and II (MHC-I and MHC-II), and immunoglobulin M and T (IgM and IgT). The Mx gene was analysed as a control gene since *a priori* it was deemed unlikely to be

differentially regulated in the gills of AGD affected fish.

2.3. Materials and Methods

2.3.1. Experimental animals

All fish used in this trial were approved for experimentation by the University of Tasmania, Australia (Animal Ethics Committee Permit No. A0011594). Seawater-acclimatised Atlantic salmon (*n* = 40), *S. salar,* weighing approximately 130 g, were held in two 500 L recirculating sea water tanks (National Centre for Marine Conservation and Resource Sustainability, University of Tasmania, Launceston, Australia) each with a 500 L pump and external biofilter and stocked at 20 fish per tank (5.2 kg m^3). Seawater was UV-irradiated and 0.2 μm (nominal) filtered. Water quality was checked every two days and salinity maintained at 35‰, temperature at 16 ± 0.5˚C, pH at 7.8-8.1 by addition of a marine pH buffer (carbonate hardness generator, Aquasonic, Wauchope, Australia) and nitrogenous wastes controlled by biofiltration. The total ammonia, nitrite and nitrate were monitored every two days using aquaria test kits (Aquarium Pharmaceuticals, Chalfont, PA, USA). Approximately 10% of the seawater was changed every week, and the fish were fed twice a day a commercial ration in a rate of 1.75% of the body weight per day. Fish were exposed to ambient photoperiod.

2.3.2. *Isolation and infection trial with N. perurans*

After a 2 week acclimation period, one group of fish was infected with *N. perurans* by inoculating 2000 trophozoites L^{-1} to the seawater. Amoebae trophozoites were isolated from the gills of AGD affected Atlantic salmon (Aquaculture Centre, University of Tasmania) according to the method of Morrison et al., 2004 and their identity confirmed by qPCR (Bridle et al., 2010).

2.3.3. Sample collection

At 0, 5 and 10 days post-inoculation (p.i.) with *N. perurans* 5 fish from the control (AGD–

unaffected) and AGD–affected tanks were euthanised in a 25 L tank with a lethal dose of anaesthetic at 5 g L^{-1} Aqui-S® (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Half of the gill basket was collected, rinsed with filtered seawater and immediately placed in 25 mL of an RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2) to preserve RNA integrity. Samples collected in RNA preservation reagent were temporarily stored at 4˚C (until biopsy punching) before the definitive storage at -20˚C. For histological analysis the second half of the gill basket was dissected and fixed in seawater Davidson's fixative (95% ethyl alcohol, 10% seawater formalin, Glacial Acetic Acid and distilled water) for 1 h then transferred to 25 mL RNA preservation reagent and stored at -20˚C.

2.3.4. Selection of gill area and gill biopsy punching

The second left gill arch was dissected and photographed with a macro camera on both sides with a scale as a landmark. The gill arch was divided into three gill segments (dorsal, medial and distal) (**Fig. 2.1.: A**) and the dorsal area was chosen as the area of interest according to the results obtained by a previous study (Adams and Nowak, 2001), which showed that AGD lesion numbers in the Atlantic salmon were significantly higher in the dorsal region of the second left gill arch relative to medial and ventral regions. Punches were carried out with a 2 mm biopsy punch in the selected area of the gill arch following the order shown in **Fig.2.1.: B**. Each biopsy punch was transferred to a 0.5 mL tube and stored at -80˚C until RNA extraction.

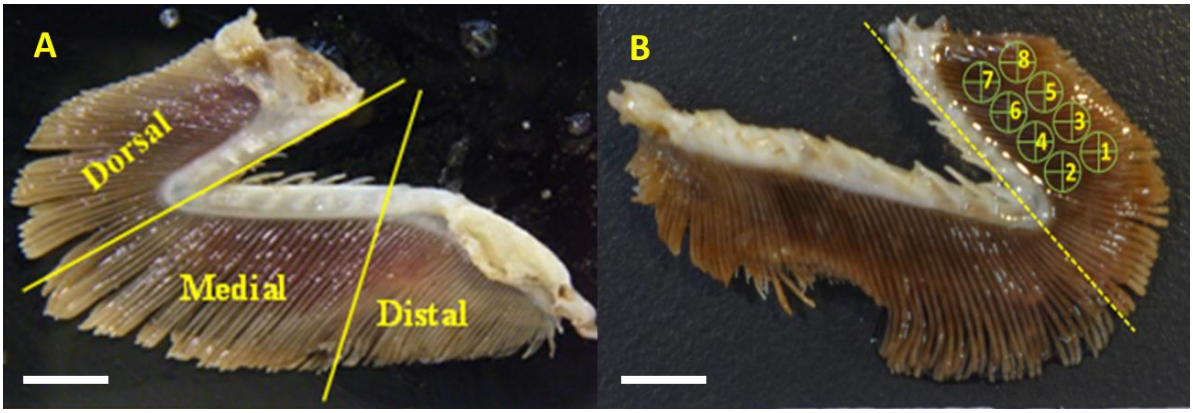

Fig. 2.1. Gross image of a gill arch with the three areas (dorsal, medial and ventral) identified (A) and the selected biopsy punched area (bar = 5 mm) (B).

2.3.5. RNA extraction from gill biopsy punches and cDNA synthesis

Gill biopsy punches from 5 AGD-unaffected and 5 AGD-affected Atlantic salmon per sample time point (80 biopsy punches per sample time point, 40 from AGD-unaffected and 40 AGD-affected fish) were individually weighed to confirm that each biopsy punch had the same weight (~ 2 mg). Total RNA was extracted from each RNA preservation reagent stabilised tissue sample with 500 µL nucleic acid extraction buffer (4M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7.5) containing 20 U of proteinase K (Bioline) per sample and homogenised manually with a micro-tube pestle. After protein digestion, cellular debris and detergent were removed by centrifugation in 3 M ammonium acetate at 14,000 × g for 5 min at 18°C, and nucleic acids were recovered by isopropanol precipitation at 16,000 × g for 10 min at room temperature followed by an ethanol wash of the nucleic acid pellet. Complete removal of DNA was ensured by treatment with 4 units Baseline-Zero DNAse (Epicentre) for 30 min at 37°C before the DNAse was removed and the RNA re-precipitated in 2.5M LiCl and washed twice in 70% ethanol. The DNA-free total RNA was resuspended in 10 µL RNAase-free water (with 0.1% Triton X-100), quantified using a Qubit fluorometer (Invitrogen) and an aliquot run on a 1% Agarose "bleach gel" (Aranda et al., 2012) stained with GelRed (Biotium) to verify integrity of the total RNA. All extracted RNA samples had well-defined 28S and 18S rRNA bands, no sign of degradation, and a 28S band approximately twice as intense as the 18S band. After RNA extraction 1µg total RNA was reverse transcribed into cDNA using a cDNA Synthesis Kit (Bioline) with Oligo $(dT)_{18}$ primer mix as outlined by the manufacturer. To confirm that contaminating trace amounts of genomic DNA were not present after RNA extraction and DNAse treatment, controls lacking reverse transcriptase were also run.

2.3.6. Quantitative real -time PCR and data analysis

qPCR was performed on a CFX Connect Real-Time PCR detection system (Bio-Rad) using SYBR green chemistry to measure the differential expression of the target genes and primer sequences listed in **Table 2.1.**. Each PCR reaction consisted of 2× SensiFast™ +SYBR® mastermix

(Bioline), forward and reverse primers (400 nM each), and 2 µL cDNA template (1 µg RNA) in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicate and cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C, 10 s at 60°C, and 10 s at 72°C. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity. Data analysis was carried out using R-studio IDE v0.97 implementing the cm3 mathematical model within the qPCR library package v1.3-7.1 that describes the entire reaction, the influences of baseline adjustment errors, reaction efficiencies, template abundance, and signal loss per cycle (Carr and Moore, 2012). The geometric mean of the two reference genes elongation factor 1α (EF1α) and beta actin (β-actin) was used to normalise the results which were then scaled to the value of the AGD unaffected group. The qPCR data were analysed with qBase plus software as described by (Hellemans et al., 2007) where mRNA expression was normalised using the geometric mean of expressions of the two reference genes ($M<1$; $CV<0.5$) (Bridle et al., 2006a; Ingerslev et al., 2006; Vandesompele et al., 2002). Fold induction or repression relative to the AGD-unaffected group was assessed for each gene using a Mann-Whitney two-tailed U-test following a $\log_{10}$ transformation as suggested by a previous study (Hellemans and Vandesompele, 2011).

Spearman's correlation analyses was used to measure the association between CD4 and CD8 expression with the TCR-α chain mRNA gene expression (**Fig. 2.5.**). Statistical significance for both analyses was set at $P < 0.05$.

2.3.7. Assessment of AGD severity for each gill biopsy punch

AGD severity for each gill biopsy punch was assessed from an evaluation of the percentage of filaments displaying gross AGD-like lesions from macro-photos of both sides of the gill arch. Punches were classified according the following model (**Fig. 2.2.**):

- AGD severity (-): no lesion
- AGD severity (+): light lesions, 1-3 affected gill filaments and lesion area 10-30%

- AGD severity (++): medium lesions, 3-5 affected gill filaments and lesion area 30-50%
- AGD severity (+++): severe lesions, >5 affected gill filaments and lesion area > 50%

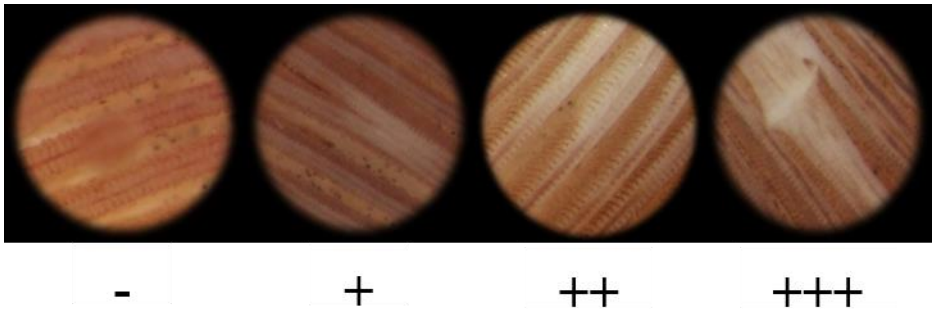

Fig. 2.2. Macro-photos of punches (AGD-affected) used to assess the AGD severity. From left to right no lesions (-), light lesions (+), medium lesions (++) and severe lesions (+++).

Gene target	Primer designation	Primer sequence (5' – 3')
β-Actin	Actin-F	GTGTGACTCGTACATTAG
	Actin-R	TAGAGGGAGCCAGAGAGG
EF1-α	EF1α-F1	TGATTGTGCTGTGCTTAC
	EF1α-R1	AACGCTTCTGGCTGTAGG
IL-1β	IL-1β-F1	AAGACACTGTTACCTACA
	IL-1β-R1	ATACCTCCAGATCCAGAC
TNF-α	TNFα-F1	CCATACATTGAAGCAGATT
	TNFα-R1	CAGCGGTAAGATTAGGATT
TCR-α chain	TCRα chain-F1	GTCTGACTCTGCTGTGTA
	TCRα chain-R1	GTGTAGTAGGATGGCTCAT
CD8	CD8-F1	AAGACAACGCTGGAATGG
	CD8-R1	TATCTGCTCCTCGCTGAA
CD4	CD4-F1	TGTTTCTGGATTCCTTTCAAT
	CD4-R1	CCATACACAAGCACATCTC
IgM	IgM-F	TGAGGAGAACTGTGGGCTACACT
	IgM-R	TGTTAATGACCACTGAATGTGCAT
IgT	IgT-F	TGCTCAGTCCGTCTCTCT
	IgT-R	ATAATTGTCTTCGCCCACCTT
MHC-I	MHCI-F1	ATTGTCCTCATCATTGTAG
	MHCI-R1	TTCTTGCTCTTCTTCTTC
MHC-II α	MHCIIα-F1	GAACATTCTTCCTCATCA
	MHCIIα-R1	TGCCATCTACACTCTATA
Mx	Mx-F	GGCATCAGATAACAGAACATC
	Mx-R	CGCACCTTCTCTTCGTAA

Table 2.1. Gene-specific primers used to amplify Atlantic salmon pro-inflammatory and immune regulatory genes.

2.4. Results

2.4.1. Infection

An examination of the gills of *N. perurans*-affected fish sampled at 5 and 10 days p.i. showed typical AGD-associated gross pathology (**Fig. 2.3.**). Numerous small multifocal pale lesions were observed on both sides of all the examined gill arches. On day 10 an increase in the number and size of lesions was observed. No signs of lesions were visible upon gross examination of the gills of the uninoculated control fish at 0, 5 and 10 days p.i. (**Fig. 2.3.**). Gill swabs were processed according to Bridle et al., 2010 and were confirmed positive by qPCR for *N. perurans* from the gills of AGD-affected fish and negative from AGD-unaffected fish.

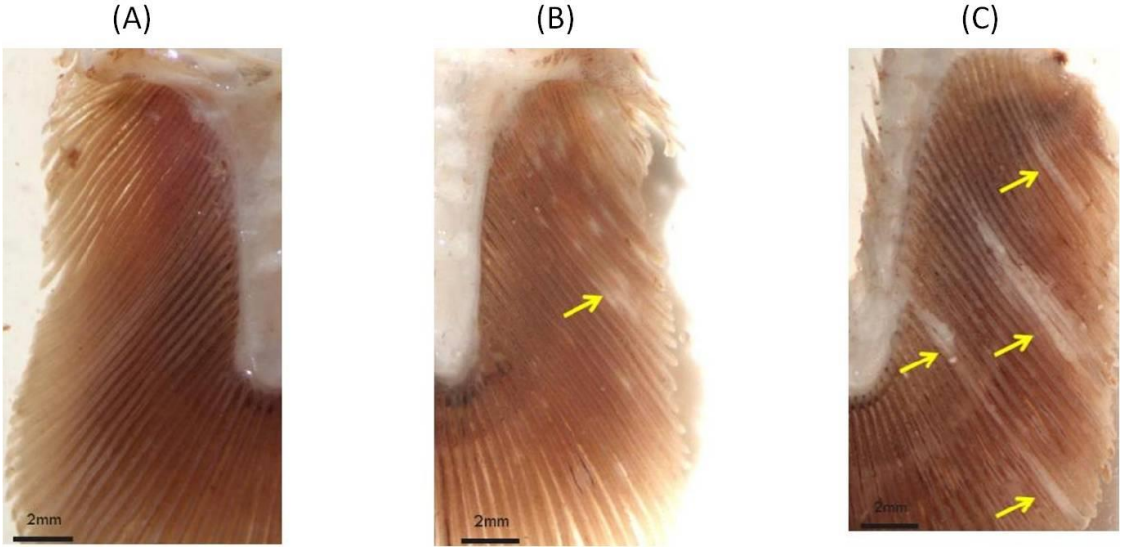

Fig. 2.3. Gross images of representative uninfected (control) (A) and AGD-affected Atlantic salmon gills at days 5 (B) and 10 (C) p.i. Typical pale multifocal mucoid lesions (arrows) associated with AGD are evident in gill images of AGD-affected fish at 5 (B) and 10 (C) days p.i.

2.4.2. Gill gene expression day 10 p.i.

qPCR showed that all the genes analysed apart from the Mx gene were up- regulated in AGD-affected gills (AGD +) (**Fig. 2.4.: A–I**). In particular, the mRNA expression of the pro-inflammatory cytokine IL-1β was significantly increased in AGD-affected fish and had 6.9 fold the IL-1β mRNA level of AGD-unaffected fish (**Fig. 2.4.: A1**). Of the AGD-affected fish the gill punches with signs

of lesions (lesion +) had 9.1 fold the IL-1β mRNA expression of the AGD-unaffected fish (**Fig. 2.4.: A2**). There was a statistically significant increase in IL-1β mRNA expression in lesion positive biopsy punches compared to biopsies with no lesions (**Fig. 2.4.: A2**). Furthermore, in punches classified as severe, IL-1β mRNA expression was most strongly up-regulated at 27.9 fold the IL-1β mRNA level of the AGD-unaffected group and declined to a 6.8 fold increase in punches classified as medium and then further declined to a 4.6 fold increase in the light classification (**Fig. 2.4.: A3**). The mRNA expression pattern of TNF-α, another pro-inflammatory cytokine, was similar to IL-1β showing an up-regulation mainly in punches classified as severe and there was a significant difference between biopsies with and without lesions (**Fig. 2.4.: B1, 2, 3**). Although the remaining immune-related genes analysed (TCR-α chain, CD8, CD4, MHC-I, MHC-II, IgM, IgT) were up- regulated in AGD-affected gills (AGD +) they showed no statistically significant difference in mRNA expression between biopsies with and without lesion in the AGD-affected gills. However, biopsies from the AGD-affected gills all had increased expression in punches classified as light, medium and no lesion (**Fig. 2.4.: C–I**). In particular, CD8 mRNA expression was strongly up-regulated in AGD-affected gills (12.4 fold the CD8 mRNA level of AGD-unaffected) and in biopsy punches classified as light (17.5 fold AGD-unaffected), medium (11.7 fold AGD-unaffected) and no lesion (11.4 fold AGD-unaffected) (**Fig. 2.4.: D1, 2, 3**). In comparison, the CD8 mRNA expression in the biopsy punches classified as severe was not significantly different than that of the AGD-unaffected fish. Furthermore, a correlation analysis between CD8-α and TCR-α showed that the mRNA expression of these two genes was strongly positively correlated (r = 0.9) (**Fig. 2.5.: L**). Interestingly, correlation analysis between CD4-α and TCR-α expression showed that there was no significant correlation (r = 0.3) (**Fig. 2.5.: M**). qPCR analysis showed that the expression of the studied genes in unaffected Atlantic salmon gills (AGD -) remained constant, as no significant change in expression of any of the genes was found (**Fig. 2.4.: A–I**).

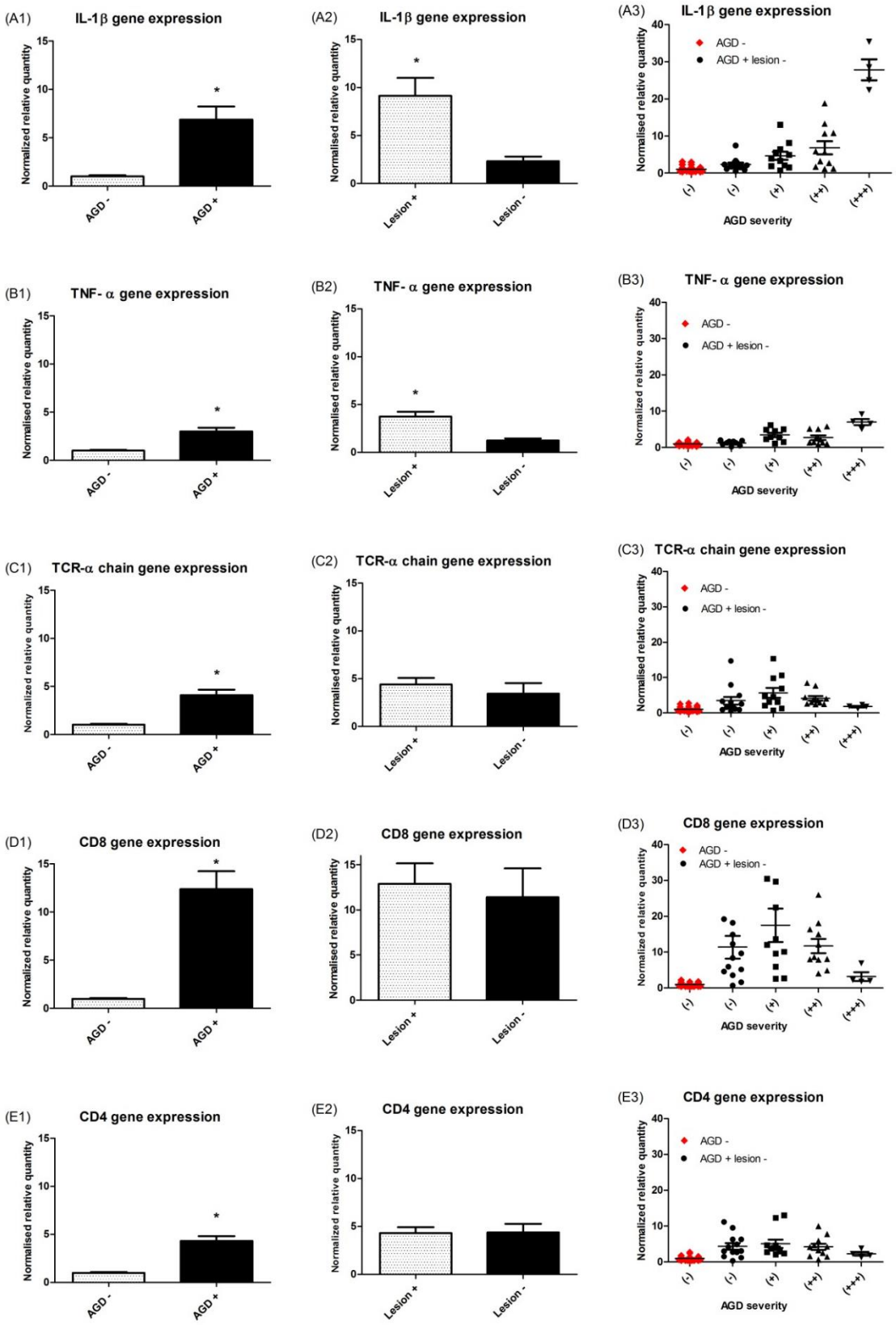

Fig. 2.4. qPCR analysis of pro-inflammatory and immune related gene expression in gill samples from AGD-affected and unaffected (control) Atlantic salmon at 10 d p.i. (A-I). qPCR analysis of Mx gene shows no significant difference between unaffected and AGD-affected gill samples (J1,2,3). Products were resolved and visualised on a GelRed-stained gel. The mRNA gene expression is relative to the geometric mean of the two reference genes EF1-α and β-actin. Bars represent mean values (+S.E.). Asterisk (*) denotes statistically significant up-regulation in target gene expression relative to the unaffected control at the same time p.i. ($P < 0.05$).

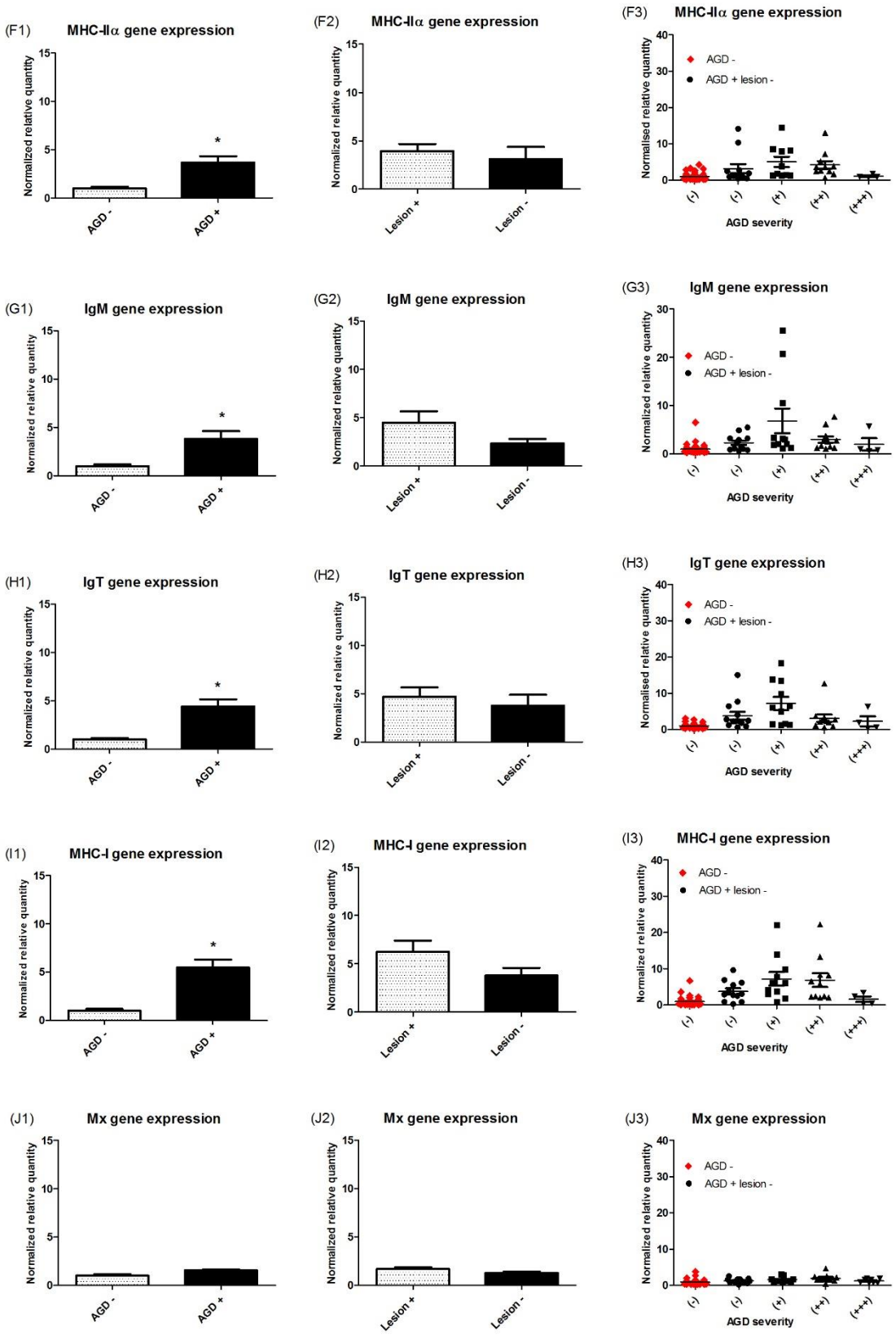

Fig. 2.4. Continued.

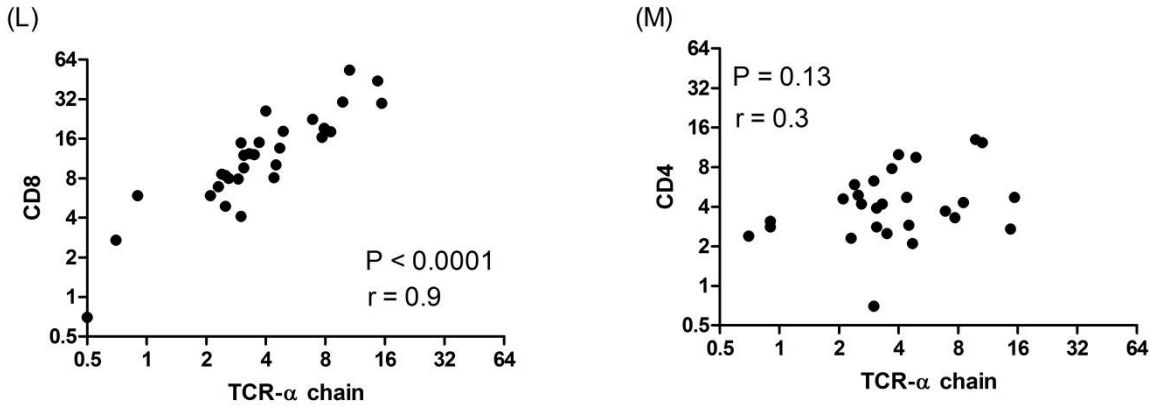

Fig. 2.5. Correlation analysis between CD8 and TCR-α chain mRNA gene expression (L) and between CD4 and TCR-α chain (M). The mRNA gene expression is relative to the geometric mean of the two reference genes EF1-α and β-actin. Correlation is positive in (L) ($P < 0.0001$ and Spearman r = 0.9).

2.5. Discussion

Based on gene expression data this study demonstrates that the parasite *N. perurans*, the causative agent of AGD, elicits a classical inflammatory response in AGD-affected Atlantic salmon, *S. salar*, and for the first time provides strong evidence of the infiltration and involvement of immune cells in AGD-affected gills. Although previous studies have reported an increased transcription of the pro-inflammatory cytokines IL-1β (Bridle et al., 2006a; Loo et al., 2012; Morrison et al., 2007; Young et al., 2008a) and TNF-α (Morrison et al., 2012) in AGD-affected Atlantic salmon there has been no conclusive evidence until now of cellular infiltration which is fundamental to most inflammatory processes initiated by pathogens or host damage. Moreover, the previously reported increased transcription of the pro-inflammatory cytokine IL-1β and TNF-α may have been explained by a possible increased expression of these two cytokines by cells already present in the gill, mainly resident immune cells and in the case of IL-1β, epithelial cells (Bridle et al., 2006a). In the present study and as previously reported in other studies (Morrison et al., 2007; Morrison et al., 2012; Young et al., 2008a) the increased expression of these two pro-inflammatory genes was more pronounced in the gills of AGD-affected fish that constituted a higher percentage of lesion to normal gill. Herein we further identified that the mRNA expression of the pro-inflammatory cytokine IL-1β was greater in

areas of the gill with a high percentage of AGD-like lesions which were classified as severe (**Fig. 2.4.: A3**). This is not surprising since epithelial cells and fibroblasts (Chaves-Pozo et al., 2003; Chaves-Pozo et al., 2004) are sources of IL-1β expression and lesions caused by *N. perurans* are mainly constituted of proliferating undifferentiated epithelial cells (Adams and Nowak, 2001; 2003; Adams et al., 2004). While not discounting the involvement of resident immune cells, namely macrophages, the likely involvement of the hyperplastic epithelia is supported by our previous finding using *in situ* hybridisation that showed IL-1β mRNA positive cells in AGD-affected Atlantic salmon gills were localised to the hyperplastic squamous epithelium lining the surface of the AGD lesions (Bridle et al., 2006a). Furthermore, previous studies on parasitic infections of fish found that in rainbow trout, *Oncorhynchus mykiss*, IL-1 is secreted by skin epidermal cells during parasitic infection with the ectoparasite *Gyrodactylus derjavini* and that this cytokine is crucial for the initiation of the anti-*G. derjavini* response (Buchmann and Bresciani, 1998; Buchmann, 1999).

Of greatest impact is our finding that AGD-affected gills displayed an increased mRNA expression of cellular markers of immune cells, most notably professional antigen presenting cells (MHC-II, CD4), B cells (IgM, IgT, MHC-II) and T cells (TCR, CD4, CD8) (**Fig. 2.4.: C–I**). The increased transcriptional expression of these cellular markers in the gills of AGD- affected fish provides strong evidence of the infiltration and involvement of a cellular immune response and the possible involvement of an antibody response from B cells. Moreover, the results of the correlation analysis (**Fig. 2.5.: L–M**) showed that the T-cells within the AGD-affected gills are mainly constituted of CD8+ cells and not CD4+ T-cells. This finding is of great importance to the possible implementation of immunoprophylactics. It also suggests that the cellular response to AGD is not only important but a possible protective response that can be exploited by immunoprophylaxis. Although speculative it is possible that the limited but increased relative protection to AGD afforded by CpGs in a previous study in our lab (Bridle et al., 2003) was the result of these known Th1 immunostimulants shifting the immune response towards or strengthening an existing cell-mediated response.

In a recent review by Nowak et al., 2014 evidence of cellular involvement in AGD is shown to be lacking apart from a limited number of studies using antibodies specific for CD3, Ig, and MHC-II that showed that CD3 and Ig were present at very low levels in AGD lesions while MHC-II was more abundant in AGD lesions (Morrison et al., 2006b). A lack of antibodies specific to immune markers in fish has resulted in the majority of immune response studies in fish occurring at a molecular level and involving transcriptional profiling. A common finding amongst these transcriptional studies is the apparent down-regulation of immune genes in AGD lesions compared to normal gill tissues of AGD-affected fish (Wynne et al., 2008a). In particular, contrary to previous studies (Morrison et al., 2012; Young et al., 2008a) we observed an up-regulation of the mRNA expression of the immune related genes MHC-I and MHC-IIα in AGD-affected gills. Therefore, by implementing a 2D qPCR approach we were able to demonstrate that the apparent down-regulation of many genes in AGD lesions in previous works (Loo et al., 2012; Morrison et al., 2006a; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a) was likely an artifact of cell type. In fact, AGD-like lesions are mainly constituted of proliferating epithelial cells, and these cells do not express the majority of immune genes. More specifically, by analysing the mRNA expression in gill biopsy punches containing varying amounts of hyperplastic epithelia we provide evidence supporting the exclusion and subsequent lack of immune cells in gill lesions while highlighting the infiltration of immune cells into the normal tissues of the AGD affected gill. Therefore, we propose that *N. perurans* does not elicit a down-regulation in an attempt to restrict the immune response as previously suggested (Wynne et al., 2008a) on the contrary it elicits an up-regulation in the gills of affected fish. Furthermore, unlike previous studies of transcriptional responses in AGD affected fish our findings are more representative of gene expression studies of other ectoparasitic infections such as the infection of rainbow trout with the *Ichthyophthirius multifiliis* and *G. derjavini* that also showed the parasite induced expression of several pro-inflammatory cytokines and immune related genes (Lindenstrom et al., 2004; Lindenstrøm et al., 2003; Sigh et al., 2004a; b).

The results obtained during this study most notably the increased expression of TCR mRNA

that was strongly correlated to CD8 mRNA, suggests the infiltration of T-cells and highlights the importance of CD8+ T-cells in AGD. This and the increased expression of cellular markers associated with antigen presenting cells and B-cells not only significantly improve our understanding of the immune response during AGD and parasitic infections in fish but are of utmost importance to the potential development of immunoprophylactics to fish parasites.

CHAPTER 3

IMMUNE GENE EXPRESSION IN THE GILLS OF ATLANTIC SALMON (*SALMO SALAR* L.) FOLLOWING EXPERIMENTAL REINFECTION WITH *NEOPARAMOEBA PERURANS*

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3.1. Abstract

Amoebic gill disease (AGD) is caused by *N. perurans* and represents a significant threat to Atlantic salmon marine farming in several countries worldwide. Sequential natural reinfection with *N. perurans* after treatment occurs after the first AGD outbreak during the grow-out phase of Atlantic salmon culture. Little is known about the immune response of Atlantic salmon following reinfection with *N. perurans.* In a previous single exposure study, using gill biopsies with various severity of AGD-lesion, we reported that the immune signalling in AGD-affected gills was highly dependent on the ratio of normal to hyperplastic gill tissues. Here, following experimental reinfection we investigated the transcriptional immune response in the gills of AGD-affected Atlantic salmon. Furthermore, we reported the inflammatory and immune response following a single exposure to *N. perurans* during late infection and compared it to the reinfected fish. Fish groups were selected based on the gross gill scores carried out during the trial. Two gill biopsies were collected from each AGD-affected individual, one with no lesion and one partly including AGD-lesion. Furthermore, gill biopsies were collected from uninfected controls. Pro-inflammatory and immune-related genes were studied at the transcription expression level using a qPCR. Targeted immune genes included IL-1β, TCR-α chain, CD8, CD4, MHC-IIα, MHC-I, IgM and IgT. Histopathology and image analysis were used to assess the severity and to verify the reliability of the gross gill score as AGD severity assessment method to select fish groups for gene expression studies. Overall the expression at the mRNA level of the immune and pro-inflammatory genes analysed showed little change in AGD-affected gills of experimentally reinfected fish and of fish exposed once to *N. perurans*.

3.2. Introduction

The marine amphizoic amoeba *N. perurans* is the confirmed etiological agent of amoebic gill disease (AGD) (Crosbie et al., 2012; Young et al., 2007). AGD is the most significant health problem affecting the production of Atlantic salmon, *S. salar* L. in Tasmania, Australia (Munday et al., 1990)

and a growing concern to the culture of other salmonids and marine fish species worldwide (Bird et al., 2006; Bustos et al., 2011; Crosbie et al., 2010; Kent et al., 1988; Kim et al., 2005a; Nylund et al., 2008; Oldham et al., 2016; Steinum et al., 2008; Young et al., 2008c). Clinical signs of AGD include white raised patches on the gills, excessive mucus production and respiratory distress (Munday et al., 1990). Grossly visible gill lesions are dominated by proliferative epithelial cells resulting in extensive lamellar fusion and formation of large vesicles that sometimes entrap amoebae (Adams and Nowak, 2001; Adams, 2003; Adams et al., 2004; Munday et al., 1990; Roubal et al., 1989). The only commercially viable treatment for the Tasmanian salmon industry to manage AGD outbreaks is to bath affected stocks in freshwater (FW) (Parsons et al., 2001). More recently, hydrogen peroxide was shown to be effective *in vivo* (Adams et al., 2012) and it is used commercially in the northern hemisphere (Rodger, 2014). FW bathing or H_2O_2 treatment represent a substantial cost for the industry since they are particularly labour intensive and require significant infrastructure (Munday et al., 2001; Parsons et al., 2001). Presumptive diagnosis of AGD by gross examination of the gills is confirmed by histopathology and molecular techniques such as conventional PCR (Young et al., 2008b) and qPCR (Bridle et al., 2010). To date several attempts to prevent the spread of AGD in farmed Atlantic salmon have been undertaken (Oldham et al., 2016), particularly previous research was focused on the formulation of diets (Dick, 2012) or the use of immunostimulants (Bridle et al., 2003; Bridle et al., 2005; Nowak et al., 2004; Powell et al., 2007; Zilberg et al., 2000) to boost the fish immune system, selective breeding of resistant lineages (Kube et al., 2012; Taylor et al., 2007), and vaccine development (Cook, 2008; Valdenegro-Vega et al., 2015a; Zilberg and Munday, 2001). Furthermore, numerous studies aimed at increasing our knowledge about the innate and adaptive immune host response to *N. perurans* were carried out (Nowak et al., 2014).

During the marine phase of Atlantic salmon production, fish are usually exposed to multiple infections by *N. perurans*. Previous field and experimental reinfection studies mainly focused on the investigation of acquired resistance to AGD (Clark and Nowak, 1999; Findlay et al., 1995; Findlay and Munday, 1998; Gross et al., 2004a; Taylor et al., 2009; Taylor et al., 2010; Vincent et al., 2006)

and histopathological progression of reinfection (Adams and Nowak, 2004). The immune gene expression studies of Atlantic salmon infected with *N. perurans* focused on a single infection event and overall, reported a down-regulation of the transcriptional immune response with the exception of an increase of the pro-inflammatory cytokine IL-1β at 12, 14, 25, 36 and 38 days post-exposure (Bridle et al., 2006a; Loo et al., 2012; Morrison et al., 2007; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a). However, a more recent work showed an up-regulation of various immune cell markers such as MHC-I, MHC-II, IgM, IgT, TCR, CD4 and CD8 at 10 days post-exposure at the periphery of the hyperplastic epithelia forming the AGD-lesions (Pennacchi et al., 2014).

Up to now there is only one study that described the transcriptional immune response in AGD-affected Atlantic salmon gills following multiple experimental infections (Valdenegro-Vega et al., 2015b). The authors of this previous experimental reinfection observed an increase close to 2-fold of IgM transcription (Valdenegro-Vega et al., 2015b). In particular, an increased level of IgM expression was detected in AGD-affected gills with AGD-lesions, whereas AGD-affected gills with no AGD-lesions showed no changes in IgM expression (Valdenegro-Vega et al., 2015b). Other immune related genes such as IgT, TCR and CD8 were analysed in the same study, however no difference in the expression was detected (Valdenegro-Vega et al., 2015b). Furthermore, following four successive exposures to *N. perurans*, there was no significant difference in the levels of IgM in fish plasma or skin mucus (Valdenegro-Vega et al., 2015b).

Based on our previous study (Pennacchi et al., 2014) following a single infection with *N. perurans* that provided evidence of the infiltration and involvement of immune cells in AGD-affected gills with a small percentage of hyperplastic gill tissues, the main aim of this study was to investigate the transcriptional immune response in the gills of AGD-affected Atlantic salmon following experimental reinfection with *N. perurans* and with differing gill pathology. In particular we investigated the immune signalling in gill biopsies, from AGD-affected fish, partly including hyperplastic gill tissues and samples with no AGD-lesion. Additionally, due to the lack of information

about immune-related markers during long-term infection with the amoeba, we intended to describe the transcriptional immune response in the fish gills following a single exposure to *N. perurans* during long-term infection (23 days post-exposure). Specifically, we compared the immune gene expression following a single exposure during late infection to the immune transcriptional response to reinfection of AGD-affected gills. In this work the targeted inflammatory and immune related genes were: interleukin-1 beta (IL-1β), T cell receptor alpha chain (TCR-α chain), cluster of differentiation 8 alpha (CD8-α), cluster of differentiation 4 alpha (CD4-α), major histocompatibility complex I and II (MHC-I and MHC-II), and immunoglobulin M and T (IgM and IgT). Our aims were achieved by using a qPCR. The gross gill score was used as a method to assess the AGD severity of the gross lesions during the experimental trial and to select fish groups for the gene expression. Furthermore, histopathology and image analysis were used to assess the AGD severity and verify the effectiveness of the gross gill score as method to determine the AGD severity in order to select fish groups for molecular analysis.

3.3. Materials and Methods

3.3.1. Fish and experimental conditions

The University of Tasmania, Australia, approved the use of all fish used in this trial (A0012237). All-female diploid Atlantic salmon (n = 307) weighing approximately 55 g, maintained in fresh water and never exposed to *N. perurans*, were obtained from a commercial hatchery in Tasmania (Petuna Seafoods Ltd, Cressy). After being sedated (1 $\mu l.l^{-1}$ clove oil) fish were transferred to a 4000 L twin tank freshwater recirculating system at the Aquaculture Centre (Institute for Marine and Antarctic Studies, University of Tasmania, Launceston, Australia). Each system had independent mechanical filtration, bio-filtration, foam fractionation and UV disinfection. The water quality was checked daily and maintained at 15.5 °C, salinity 0‰, pH 6.8-7.2, TA-N ≤ 1.0 $mg.L^{-1}$, NO_2 < 2 $mg.L^{-1}$, $NO_3 \leq 40$ $mg.L^{-1}$ and DO_{sat} > 85%. Fish were fed twice a day a 3 mm sinking pellet to a maximum

of 2% BW/day (Skretting Spectra & Spirit Supreme) for the duration of the trial. After one month of habituation the fish (~ weight 70 g) were sedated (30 $\mu l.l^{-1}$) and intramuscularly tagged with Passive Integrated Transponder (PIT) tags and gradually acclimated to 35 ppt. After the acclimation period the fish were sedated and randomly reallocated to either a 2000 L twin tank system or returned to the original system.

3.3.2. N. perurans isolation

N. perurans isolation was performed according to Morrison et al., 2004. Trophozoites of *N. perurans* were isolated from the gills of AGD-affected Atlantic salmon held in an ongoing infection tank located at the Aquaculture centre, University of Tasmania.

3.3.3. Experimental design

The experimental timeline and design are reported in **Fig. 3.1.** Following four weeks of acclimation period fish (average weight 181 g) in the 4000 L system were exposed for the first time to amoebae (194 cells/L). The fish in the 2000 L system were unexposed to amoebae for the duration of the trial. After 4 weeks fish gills were checked, grossly scored (**Table 3.1.**) and fish were freshwater (FW) bathed for 3 hours. The control group was handled and treated in the same way. The day after the FW bath fish were re-exposed to amoebae (158 cells/L) for the second time. After 17 days gills were checked and the fish were FW bathed. Fish were FW bathed again 1 week after the previous bath. After 2 days fish were re-exposed to amoebae (1000 cells/L) for the third time and after 3 weeks gills were checked, scored and fish transferred into a FW bath. Fish were returned to the experimental tank without adding extra amoebae and relying on reinfection from the amoebae surviving treatment (Adams et al., 2012; Gross et al., 2004b) to mimic circumstances *in situ* (Adams and Nowak, 2004). On the same day 20 naïve fish from the control tank were added to the infection system. After one month gills were checked and fish were re-exposed to additional amoebae (475 cells/L). At the end

of the trial, 4 weeks after the last challenge, all fish were sampled and the gills assessed for gross signs of pathology.

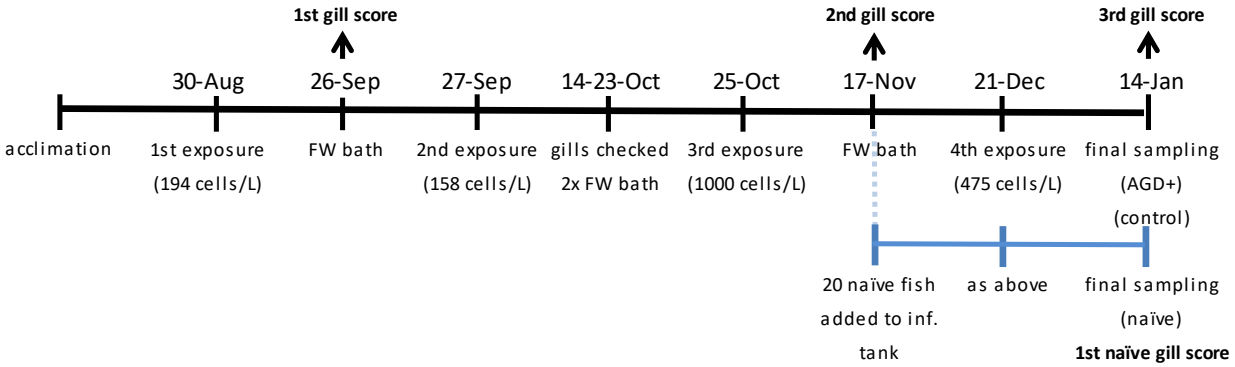


Fig. 3.1. Experimental timeline showing challenges, FW baths and times for gill scores.

3.3.4. Sample collection

At the end of the trial fish (average weight of salmon from the experimental system 397 g and from the control 516 g) were all sampled (n = 174) and euthanised in a 400 L tank with a lethal dose of anaesthetic at 5 g/L Aqui-S® (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Weight, length, gross gill score and any anomalous gross observation were recorded for each fish. To carry out the molecular analysis the third right gill arch was excised, rinsed in filtered seawater and placed in 25 mL of RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) to preserve RNA integrity. Samples were temporarily stored at 4 °C (until biopsy punching) before definitive storage at -20 °C. The left side gill arches were dissected, rinsed in filtered seawater, placed in Davidson's seawater fixative and after 24 h transferred to 70% ethanol. The second left gill arch was processed for routine histology.

3.3.5. Gross assessment of AGD severity during the trial

AGD severity was assessed during the reinfection trial categorizing the presence and extent of grossly visible lesions (**Table 3.1.**). All fish, exposed and unexposed to *N. perurans*, were assessed

at each check to ensure no signs were evident in unexposed fish as well as imparting equivalent handling procedures between groups.

Infection level	Gross pathology
Clear	no lesions observed
Light	one or more small lesions affecting one or more hemibranchs
Moderate	multiple small lesions affecting all hemibranchs
Heavy	multiple lesions affecting all hemibranchs with substantial coverage

Table 3.1. Scoring system for the assessment of gross gill lesions, used during the experiment, to quantify the level of infection on Atlantic salmon. The method semi-quantifies the clinical severity of grossly visible lesions**.**

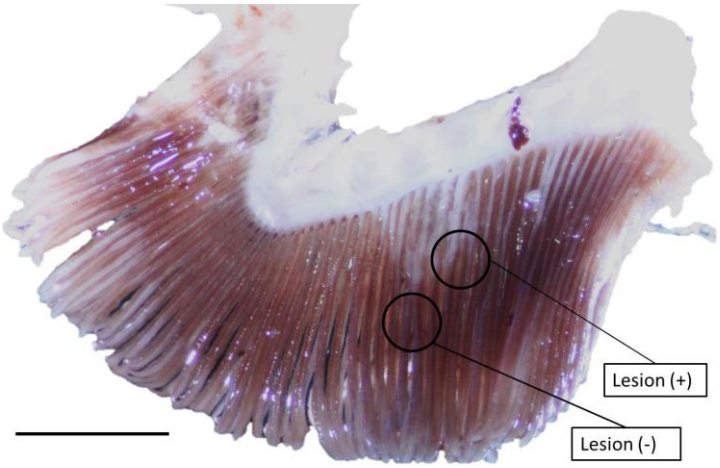


Fig. 3.2. Gross image of gill arch before punching showing areas of the gill chosen for biopsy collection: AGD lesion (+) and AGD lesion (-) (bar = 500 µm).

3.3.6. Selection of fish groups

Fish (n = 35) were chosen for biopsies according their individual gill assessment histories recorded during the trial. Based on the gill scores 4 groups were identified and selected: *light* (gills categorised as light each gill score), *moderate* (gills categorised as moderate each gill score), *clear* (gills categorised as clear at the last gill score) and *naïve moderate* (gills of previously unexposed fish infected once and categorised as moderate). In addition to these 4 categories, biopsies were collected also from unexposed fish (AGD negative and all clear at each gill score). For each category 7 fish of approximately the same size (average ± SD: weight 371.5 ± 103 g and length 306.7 ± 28 mm) were selected.

3.3.7. Histological assessment of AGD severity

One gill arch section for each fish (the same individuals as used for biopsies) was viewed under a light microscope (Olympus, Hamburg, Germany) at 400-1000x.

The histological parameters to be assessed for each group were (**Table 3.2.**):

- % of filaments with hyperplastic lesions
- % of filaments with lesions colonised by amoebae
- % of lesions colonised with amoebae
- mean lesion size (hyperplastic lamellar units – ILUs)

The mean lesion size was assessed for all groups by counting the number of ILUs) fused in each lesion (**Table 3.2.**) according to Adams and Nowak, 2001. The differences between groups for these histological parameters were assessed by a One-way ANOVA combined with a Tukey's multiple comparison test in Graphpad Prism 5.01® biostatistics software. Values were considered statistically different at P-values < 0.05. The hyperplastic lesions colonised by amoebae were considered as AGD-lesions.

3.3.8. Assessment of AGD severity using image analysis

Holobranchs (a pair of hemibranchs on the same gill arch) fixed in marine Davidson's fixative (24-48 h) were excised from the left side of the gill basket and placed in a petri dish filled with ethanol (70% v/v). The anterior and posterior aspects were photographed using a macro lens fitted to a camera (Canon EOS 550D – resolution 5184 x 3456 pixels). To assess the gross affected area a method modified from Torchareon, 2014 was used. Briefly, all images were sharpened and their contrast increased before converting them to grey scale using ImageJ (Schneider et al., 2012). The total area of gill holobranch was selected using the threshold function and expressed as pixel area. The cartilage pixel area was subtracted from the visible gill holobranch area. The focal white spots or raised patches were selected using the freehand selections function and considered as gross affected pixel area.

The percentage of gross affected area was calculated using the following equation:

$$\%\ \text{of gross affected area} = \frac{\text{Affected area } \times 100}{\text{Total gill filament area}}$$

The differences in percentage (%) of gross affected area between groups for both holobranchs, anterior holobranch and posterior holobranch were assessed by a One-way ANOVA followed by a Tukey's multiple comparison test. A Mann-Whitney two-tailed U-test was used to assess differences in % of gross affected area between anterior and posterior holobranch in each group. All percentages of the gross affected area were arcsine transformed. Before the ANOVA the arcsine transformed data were tested for homogeneity of variances by a Levene's test showing that variances were homogeneous ($P > 0.05$). All measurements of gross affected area are presented as mean (±SE). Spearman's correlation analysis was used to estimate the association between the percentage of gross affected area and mean lesion size for the second left gill arch. All data analyses were performed using Graphpad Prism 5.01 with a significance level of $P < 0.05$.

3.3.9. Gill area selection for biopsies

For all groups (except unexposed and *clear*), two biopsies were collected from each fish/gill arch using a 2 mm biopsy punch. One biopsy classified as lesion (-), was taken from a non-lesion

area of the gill arch (**Fig. 3.2.**). The second biopsy, classified as lesion (+), was collected from AGD-lesion area (small percentage of lesion included in the biopsy) of the same gill arch used to collect lesion (-) biopsies (**Fig. 3.2.**). For the control and *clear* groups one 2 mm biopsy for each fish/gill arch was collected from the distal area of the gill arch.

3.3.10. RNA extraction and cDNA synthesis

Gill biopsies were weighed to confirm that each biopsy had approximately the same weight (~ 3 mg). The total RNA was extracted from RNA preservation reagent stabilised samples using 400 µL nucleic acid extraction buffer (4M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 8.2) containing 20 U of proteinase K (Bioline, NSW, Australia) per sample and homogenised manually with a micro-tube pestle on ice. After protein digestion (30 min), samples were centrifuged at 4 °C in 7.5 M ammonium acetate at 16,000 x g for 3 min to eliminate cellular debris and detergent. An isopropanol precipitation (1 volume added) at room temperature (RT) at 16,000 x g for 8 min was done to recover nucleic acids. RNA was resuspended in 190µL RNAase-free water and heated at 55 °C to aid resuspension. After resuspension, RNA was re-precipitated in 10 µL 7.5M LiCl and 400 µL 100% ethanol then centrifuged at 16,000 x g for 10min at RT. After centrifugation the supernatant was removed and the pellet was resuspended in 180µL RNAase-free water and heated at 37 °C to aid resuspension. After resuspension, 20µL 10x DNAse buffer was added at 37 °C. DNA was completely removed by 2 treatments with Baseline-Zero DNAse, 15 min the first and 30 min the second (2 units each) at 37 °C. After DNAse treatment, RNA was re-precipitated in 10 µL 7.5M LiCl and 400µL 100% ethanol, centrifuged at 16,000 x g for 10 min at RT and finally washed 1x with 70% ethanol. The DNA-free total RNA was re-suspended in 15 µL RNAase-free water + dTT, quantified using a Qubit fluorometer (Invitrogen) and an aliquot electrophoretically separated on a 1% agarose gel to verify integrity of the total RNA. All extracted RNA samples showed no sign of degradation and had well-defined 28S and 18S rRNA bands. The 28S band was approximately twice as intense as the 18S band. After RNA extraction 1µg total RNA was reverse transcribed into cDNA using a cDNA

Synthesis Kit (Bioline) with Oligo $(dT)_{18}$ primer mix as outlined by the manufacturer. Controls, lacking reverse transcriptase, were run to confirm that genomic DNA contamination did not occur after RNA extraction and DNAse treatments.

3.3.11. Quantitative RT-PCR and data analysis

Lesion (-), control and *clear* biopsies were analysed first for gene expression using a qPCR. Lesion (+) biopsies were analysed running a second qPCR and using the same total RNA extracted from control and *clear* biopsies. A CFX Connect Real-Time PCR detection system (Bio-Rad) was used to perform qPCR. The SYBR green chemistry was used to measure the expression of the target genes (see **Table 3.2.** for primer sequences). A master mix was prepared for each PCR reaction and consisted of 2x polymerase/sybr mix (SensiFast) and forward and reverse primers (400 nM each). The mastermix (8 µL) was transferred to each column of the plate and 2 µL cDNA template (1µg of RNA) in molecular grade water was added to a final volume of 10 µL. The cycling protocol included an initial activation of DNA polymerase at 95 °C for 3 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C. All samples were run in duplicates and at the end of the cycling protocol melt curve analyses were run to ensure amplification specificity with fluorescent detection conducted at 0.5 °C intervals. qPCR runs were only considered for analyses when amplification efficiency (E) of standard dilutions ranged between 85-105% with a linear regression (r2) of 0.98-1.00. After all the runs, the cycle threshold (Ct) values were copied to an excel spread sheet and data imported into qBasePLUS® analysis software (Biogazelle, Zwijnaarde, Belgium) to carry out the qPCR data analysis as previously described (Hellemans et al., 2007). To normalise the mRNA expression, the mean of the two reference genes, elongation factor 1-alpha (EF1-α) and beta-actin (β-actin) was used (see **Table 3.2.** for primer sequences) (Vandesompele et al., 2002). The qPCR data were analysed with qBase plus software as described by (Hellemans et al., 2007) where mRNA expression was normalised using the geometric mean of expressions of the two reference genes ($M<1$; $CV<0.5$) (Bridle et al., 2006a; Ingerslev et al., 2006; Vandesompele et al., 2002).

To assess differences in the immune gene mRNA expression among the different groups analysed a One-way ANOVA was run in qBasePLUS®. When differences were detected, values were considered statistically different at *P*-values < 0.05. To show the fold induction or repression in the graphs a log2 transformation was used (**Fig. 3.5.**). Raw data and statistical results (statistical significance, geometric mean and 95% CI) obtained from qBasePLUS® were graphed by Graphpad Prism 5.01. The analysed genes were considered significantly expressed at biological level with a minimum mean of 2-fold upwardly.

Gene target	Primer designation	Primer sequence (5' – 3')
β-Actin	Actin F	GTGTGACTCGTACATTAG
	Actin R	TAGAGGGAGCCAGAGAGG
EF1-α	EF1α-F1	TGATTGTGCTGTGCTTAC
	EF1α-R1	AACGCTTCTGGCTGTAGG
TCR-α chain	TCR-α chain-F1	GTCTGACTCTGCTGTGTA
	TCR-α chain-R1	GTGTAGTAGGATGGCTCAT
CD8	CD8-F1	AAGACAACGCTGGAATGG
	CD8-R1	TATCTGCTCCTCGCTGAA
CD4	CD4-F1	TGTTTCTGGATTCCTTTCAAT
	CD4-R1	CCATACACAAGCACATCTC
IgM	IgM-F	TGAGGAGAACTGTGGGCTACACT
	IgM-R	TGTTAATGACCACTGAATGTGCAT
IgT	IgT-F	TGCTCAGTCCGTCTCTCT
	IgT-R	ATAATTGTCTTCGCCCACCTT
MHC-I	MHCI-F1	ATTGTCCTCATCATTGTAG
	MHCI-R1	TTCTTGCTCTTCTTCTTC
MHC-II	MHCIIα-F1	GAACATTCTTCCTCATCA
	MHCIIα-R1	TGCCATCTACACTCTATA
IL-1β	IL-1β-F1	AAGACACTGTTACCTACA
	IL-1β-F1	ATACCTCCAGATCCAGAC

Table 3.2. Primers used to amplify selected Atlantic salmon immune regulatory genes.

3.4. Results

3.4.1. Histopathology

Histopathological results are summarised in **Table 3.3.** The percentage of lesions colonised with amoebae showed that the group *clear* (5.7 ± 3.6) was significantly different ($P = 0.0103$) from *light* (40.9 ± 30.4) and *naïve moderate* (52.2 ± 17.7) groups (**Table 3.3.**). The mean lesion size (ILUs) showed that the group *clear* (7.5 ± 3.7) was significantly different ($P = 0.0004$) from *moderate* (17.4 ± 3.7) and *naïve moderate* (21 ± 8.3) groups and the group *light* (13.3 ± 3.2) was significantly different ($P = 0.0004$) from *naïve moderate* (21 ± 8.3) group (**Table 3.3.**). There was no significant difference between the groups for the percentage of filaments with hyperplastic lesions and percentage of filaments with lesions colonised by amoebae (**Table 3.3.**).

Histological parameters	**Clinical AGD severity at final sampling**			
	Clear	**Light**	**Moderate**	**Naïve moderate**
% filaments with hyperplastic lesions	35.9 ± 16.6	36.6 ± 14.9	39.5 ± 18.0	32.5 ± 14.2
% filaments with lesions colonised by amoebae	2.4 ± 1.9	15 ± 14.0	13.7 ± 15.6	16.3 ± 8.7
% lesions colonised with amoebae	5.7 ± 3.6^{a}	40.9 ± 30.4^{b}	36.8 ± 28.1^{abc}	52.2 ± 17.7^{c}
Mean lesion size (ILUs)	7.5 ± 3.7^{a}	13.3 ± 3.2^{ab}	17.4 ± 3.7^{bc}	21 ± 8.3^{c}

Table 3.3. Histological assessment of AGD severity for all groups. The percentage of mean ± SD values are reported. Different letters identify groups that are significantly different ($P < 0.05$).

3.4.2. Image analysis

Generally, the group classification based on the gross gill score reflected both the percentage of gross affected area calculated by the image analysis and the histological assessment of AGD severity (mean lesion size). In particular, the correlation analysis between the percentage of gross

affected area and the mean lesion size for the second left gill arch showed that there was a positive correlation (P = 0.0017 and r = 0.6) (**Fig. 3.3.**).

Significant differences were found between groups in the % of gross affected area for both holobranchs, anterior holobranch and posterior holobranch. In all situations considered, *clear* was significantly different ($P < 0.05$) from *light* and there was no significant difference between *moderate* and *naïve moderate*. Furthermore, *moderate* and *naïve moderate* were the groups most affected and significantly different ($P < 0.05$) from both *clear* and *light* (**Fig. 3.4.: A1, A2, A3**).

In the *moderate* group, the % of gross affected area in the two sides of each holobranch was significantly higher (P = 0.0114) for the posterior holobranch (7.39% ± 0.7) relative to the anterior holobranch (5.38% ± 1.0) (**Fig. 3.4.: A4**). All the other groups did not show significant differences related to arch holobranch side (data not shown).

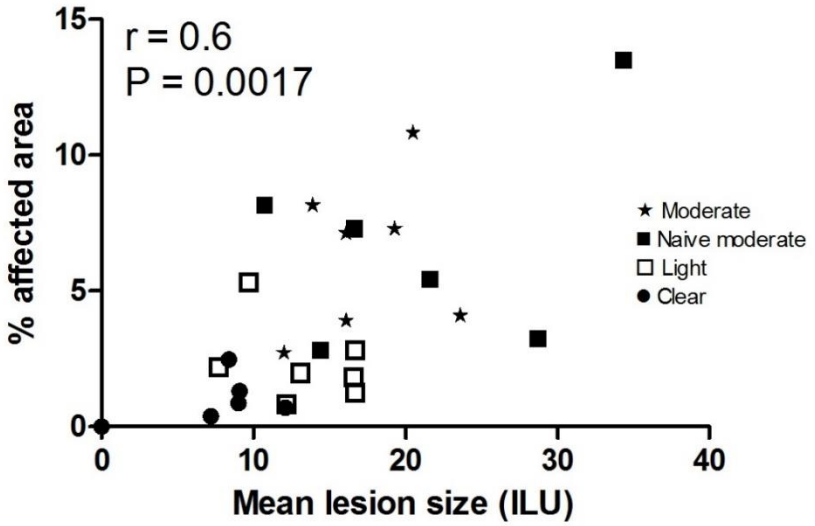


Fig. 3.3. The scatter plot shows the relationship between the percentage of gross affected area and the mean lesion size for the second left gill arch. Different symbols indicate individuals belonging to different groups assessed by gross gill score. The correlation is positive: $P < 0.05$ and Spearman r = 0.6.

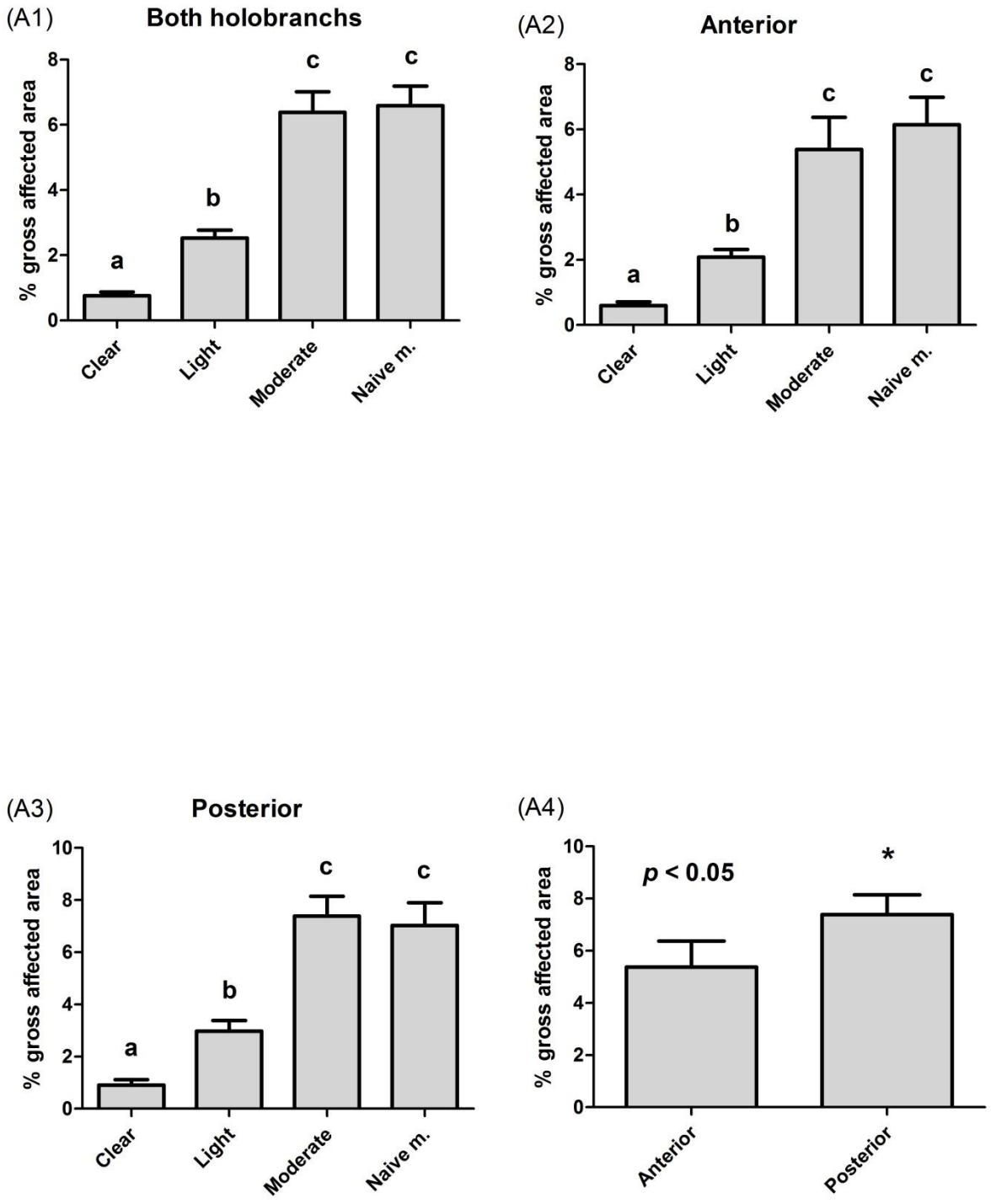


Fig. 3.4. Graphs A1-A3 represent the percentage (% mean ± SE) of gross affected area between groups. Different letters denote significantly different ($P < 0.05$) means between groups. Graph A4 reports the percentage (% mean ± SE) of gross affected area for anterior and posterior holobranch. The asterisk indicates a significant difference between the two means.

3.4.3. Gill gene expression

3.4.3.1. Lesion (+) biopsies

The mRNA expression of the immune genes TCR-α chain and CD8 was significantly lower (approximately 2-fold) in fish groups exposed to *N. perurans* and classified as *light* and *moderate* relative to control and *clear* (**Fig. 3.5.: I, J**). In particular, the *moderate* group showed an approximately 2.5-fold down-regulation of TCR (**Fig. 3.5.: I**). MHC-IIα mRNA expression was significantly lower (2-fold) in *light*, *moderate* and *naïve moderate* groups relative to control (**Fig. 3.5.: L**). Particularly, MHC-IIα showed a 2.5-fold down-regulation in *light* relative to control (**Fig. 3.5.: L**). IgM mRNA expression was significantly lower (approximately 2.5-fold) in *light* group than all other groups (**Fig. 3.5.: N**). The pro-inflammatory cytokine IL-1β in the *moderate* group was the only significantly up-regulated gene showing an approximately 3-fold up-regulation relative to the control and *clear* groups (**Fig. 3.5.: H**). No significant differences were found between any groups for CD4, IgT, and MHC-I mRNA gene expression (**Fig. 3.5.: K, M, O**).

3.4.3.2. Lesion (-) biopsies

The mRNA expression of immune gene TCR-α chain was significantly down-regulated (2-fold) in the *light* and *moderate* groups relative to unexposed group (**Fig. 3.5.: A**). The clear and *naïve* groups showed significantly lower expression of IgT mRNA relative to control, in particular for the *naïve* group IgT mRNA was approximately 2.5-fold down-regulated (**Fig. 3.5.: E**). MHC-I mRNA expression showed a significant down-regulation (2-fold) in all groups relative to unexposed fish aside from *naïve* (**Fig. 3.5.: G**). There were no significant differences between any groups for CD4, CD8, MHC-II, IgM and IL-1β mRNA gene expression (**Fig. 3.5.: C, H**).

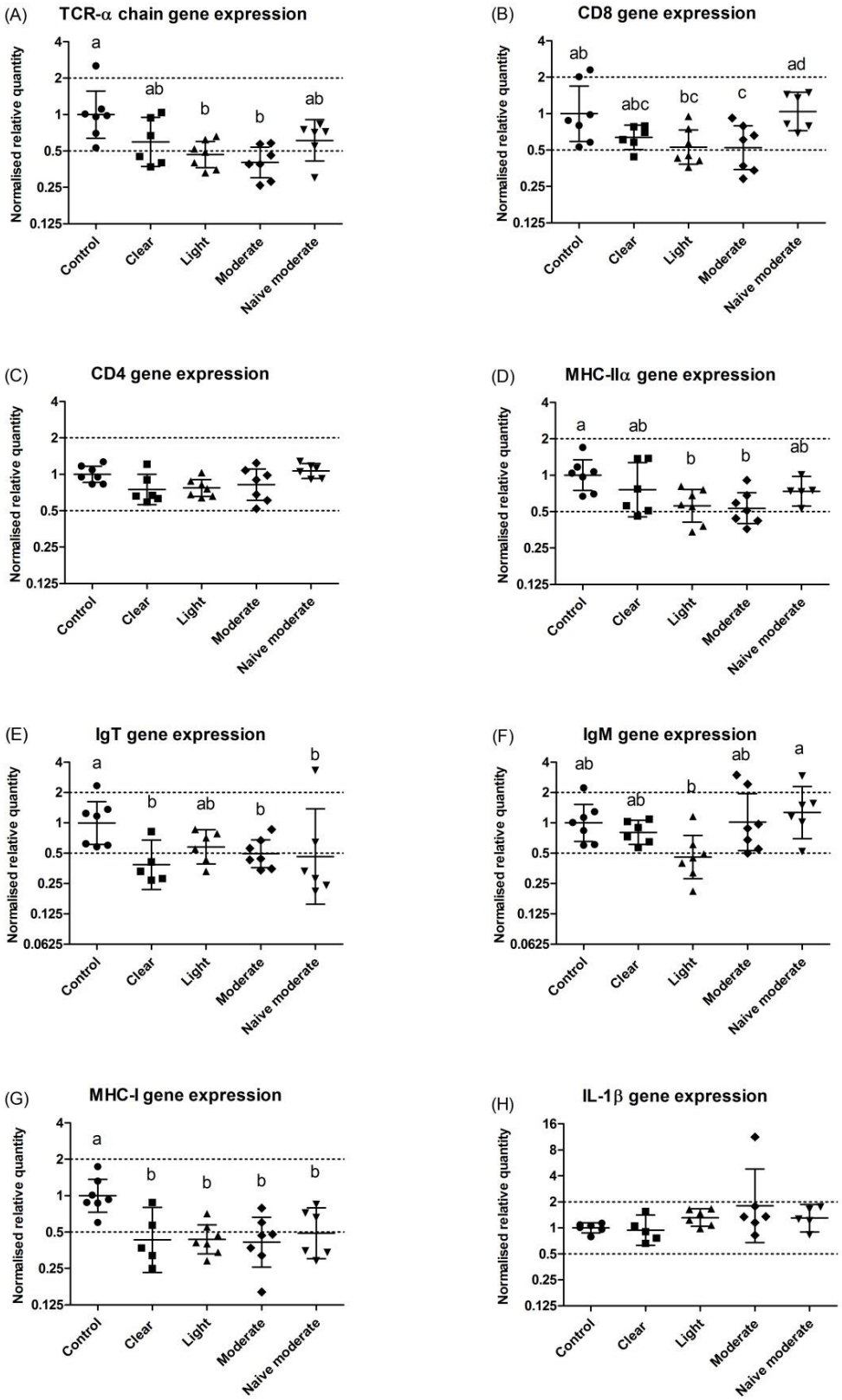


Fig. 3.5. qPCR analysis of immune related gene expression in gill biopsies obtained from Atlantic salmon exposed 4x (clear, light and moderate) to *N. perurans* and FW bathed, 1x (naïve moderate) with no FW bath given; and unexposed fish (control). The mRNA gene expression is normalised and relative to the two reference genes EF1-α and β-actin. Lines represent geometric mean values and bars 95% CI. Different letters identify groups that are significantly different ($P < 0.05$). Graphs A-H represent close to lesion type biopsies whereas graphs I-P represent lesion type biopsies.

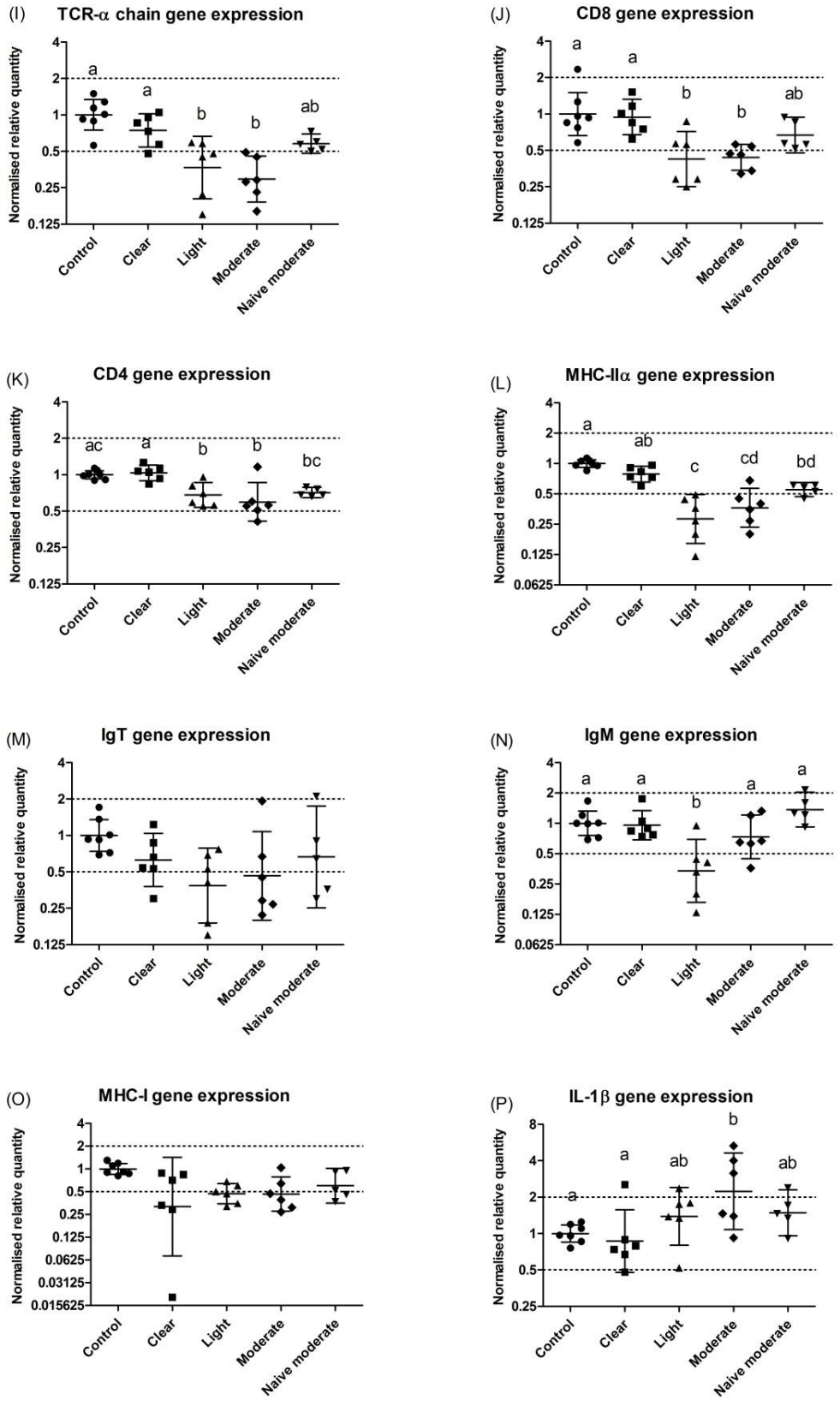


Fig. 3.5. Continued.

3.5. Discussion

The only gene that showed a slight up-regulation (2.5-fold) relative to control was the pro-inflammatory cytokine IL-1β in the gills of reinfected fish (137 days after the 1st exposure and 24 days after the last) in the group classified as *moderate* in lesion (+) biopsies. This finding is consistent with our previous results where an increased expression of the pro-inflammatory cytokine IL-1β was observed in biopsies from AGD-affected fish with a high percentage of AGD-lesion consisting of hyperplastic epithelium to normal gill (Pennacchi et al., 2014). Previous AGD trials in Atlantic salmon showed that there was an up-regulation of IL-1β (Bridle et al., 2006a; Morrison et al., 2006a; Morrison et al., 2012; Pennacchi et al., 2014) at 10, 12, 14, 25, 36 and 38 days post-exposure, particularly in more advanced AGD-lesions (Pennacchi et al., 2014). Furthermore, an increase of IL-1β expression at the infection site has been shown in other ectoparasitic infections including rainbow trout with *Ichthyophthirius multifiliis* (see Sigh et al., 2004a) and *Gyrodactylus derjavini* (see Buchmann and Bresciani, 1998; Buchmann, 1999) suggesting that this pro-inflammatory cytokine is fundamental for the host response to pathogens. The pro-inflammatory cytokine IL-1β was significantly up-regulated only in lesion (+) biopsies *moderate* group whereas the other groups, such as *light* and *naïve moderate*, showed no change in the mRNA expression. Histological observations provided a possible explanation related to the cellular composition of different stages of AGD-lesions with mature or larger AGD-lesions often showing greater mucous cell hyperplasia. Mucous cells which are the fish equivalent of mammalian goblet cells are a modified epithelial cell and are known to express IL-1β during AGD in Atlantic salmon (Bridle et al., 2006a).

Individual variability detected at the mRNA expression level in the same group is most likely due to the variation in the biopsy composition of gills assigned to a group by visually assessed gross AGD-lesion severity; in particular, changes in the types and ratio of cell populations at different developmental stages of lesions associated with AGD (Nowak et al., 2013), within each group and

most importantly within each fish. This last observation is supported by analogous findings from mammalian studies on tumour gene expression where the heterogeneity at cellular level is mirrored at the gene expression level (Korsten et al., 2016; Perou et al., 2000; Sørlie et al., 2001). The AGD-lesion, especially during repeated exposures to the parasite and FW treatments, might undergo a process of remodelling and repairing that changes according to different stages of the infection and different amoebic burden. Thus, the AGD-lesion and the closer surrounding area should be considered as a micro-environment in the AGD-affected gills where a cross-talk of various cell populations may occur and contribute to explain the variability at the transcriptional level detected in this study and also more broadly reported from gene expression studies on AGD. In support of this, previous studies on mammals showed that different regulating mechanisms are associated with tissue remodelling following inflammation at the site of injury or infection and that this process is driven by a cross-talk between epithelial, fibroblast, and immune cells (Broide, 2008; Micera et al., 2016; Wynn, 2007).

Recently, another study on experimental multiple infection with *N. perurans* carried out by researchers from our laboratory highlighted the relevance of AGD-lesion developmental stage (Valdenegro-Vega et al., 2015b). In particular, a close to 2-fold increase of IgM was observed in AGD-affected gills with AGD-lesions and no difference was found in the expression of IgT, CD8 and TCR in AGD-affected gills with no apparent lesion or with typical AGD-lesion (Valdenegro-Vega et al., 2015b). Conversely, in our study a slight decrease of IgM, IgT and TCR mRNA expression relative to control in AGD-affected gills was detected. However, both in this and in the previous study the limited differential gene expression was of questionable biological significance displaying a relatively minor fold change (approximately 2-fold). In our study the samples classified as lesion (+) had a small percentage of lesion whereas in the previous study the lesion (+) samples were only constituted of lesion (Valdenegro-Vega et al., 2015b). Moreover, in the previous experiment the fish were perfused to completely remove blood from the gills (Valdenegro-Vega et al., 2015b), thus the samples did not contain any blood. As a consequence, the contribution of blood cells including leukocytes known to express high levels of many immune-related genes was greatly

reduced. Another possible reason for the differences of the gene expression between the two studies could be related to the experimental design with the final sampling time different between the two reinfection trials. Fish in this experiment were collected 15 weeks after the first exposure (3 weeks after the last exposure) to *N. perurans* whereas fish were sampled 18 weeks after the first exposure (4 weeks after the last exposure) in the other study (Valdenegro-Vega et al., 2015b). Furthermore, the fish in these two trials were exposed to a different infection pressure. In this study the animals were exposed initially to 194 cells/L and re-exposed to 158, 1000 and 475 cells/L respectively at 4, 8, 12 weeks later whereas Valdenegro and colleagues, exposed the fish to the same initial density (150 cells/L) at 5, 8 and 14 weeks (Valdenegro-Vega et al., 2015b). Thus, the increase in IgM expression could have been transient and outside our sampling time or most likely, since the change detected in the previous study was only a slight increase (Valdenegro-Vega et al., 2015b), our biopsies had a different cellular composition. Since both this and the previous work lacked of detailed information about the sample cellular composition used for PCR analysis we were not able to compare it between the two studies.

In a previous natural reinfection trial on sea-cage reared Atlantic salmon an increased transcription of immune related genes, especially MHC-II invariant chain-like protein (close to 2-fold) and Ig light-chain (close to 5-fold) was reported (Wynne et al., 2008b). The increase was observed in the gills of fish categorised as clear at the last gill score (considered AGD-resistant) relative to fish categorised as heavy at the last gill score (considered AGD-susceptible) and to a minor extent also relative to uninfected fish (Wynne et al., 2008b). However, Wynne et al., 2008b did not describe the uninfected fish (e.g. fish size, background and rearing conditions), therefore the differences detected between resistant and uninfected fish could be a by-product of ontogeny. In contrast, we observed a slight decrease of MHC-II in AGD-affected gills of *moderate* fish relative to both *clear* and uninfected particularly in lesion (+) samples, and no difference for IgM between *moderate* and *clear* categories. However, the fold change detected for MHC-II in the previous experiment was of questionable biological significance since it was lower than 2-fold. There are a

few possible reasons for the dissimilarities at transcriptional level between the two reinfection studies. Firstly the trial design as in the previous work the fish were in sea-cages and naturally reinfected by the parasite (Wynne et al., 2008b) whereas herein the fish were experimentally reinfected, thus under controlled conditions. Second the amoebic burden was likely different as Wynne and colleagues exposed the fish to a natural amoebic load (Wynne et al., 2008b). Another difference is related to the sampling strategy and fish categories as in the previous research was not specified if the samples used for the heavy category (or AGD-susceptible) had lesions or not (Wynne et al., 2008b) making harder the comparison with our results and furthermore we used fish categorised as moderate that is with a lighter AGD-severity. Although the authors of the previous research attributed the increase of the immune gene expression in the heavy category relative to the clear category to differences between resistant/susceptible fish we suggest that the detected differences in this and the previous study are most likely caused by dissimilarities between different cell types present in the samples. This explanation is further supported by their observation of an increased IL-1β transcription in the gills of heavy infected fish relative to both clear and uninfected fish (Wynne et al., 2008b), somewhat similar to our IL-1β results from lesion (+) samples. In fact the up-regulation of this inflammatory cytokine is likely associated with the presence of the hyperplastic epithelium in moderate/heavily AGD-affected gills (Pennacchi et al., 2014).

In the present study the contribution of receding/small AGD-lesions that were not visually apparent during gross assessment cannot be ignored as it is possible that lesion (-) biopsies may have contained AGD-lesions. For instance the *clear* group did not show any sign of AGD-lesion during the gross gill score whereas histological analysis showed that the percentage of gill filaments with hyperplastic lesions was similar to the other groups and amoebae colonising the lesions were observed. Furthermore, histology showed that small AGD-lesions in the *clear* group were frequently dominated by lymphocytes. However unlike our previous study (Pennacchi et al., 2014) this finding implies a recruitment of immune cells to the infection site of the AGD-affected gills. Additionally, lesions in the *clear* group were detected with the image analysis.

A decreased transcription of the immune genes IgT, MHC-I and MHC-II was previously reported in the gills of naïve fish 36 days after single exposure to *N. perurans* (see Young et al., 2008a). This decrease of the immune gene expression is somewhat in agreement with the results obtained in the present study for the fish with a single exposure (*naïve moderate* group) to the amoeba. However, the slight decrease observed in some of the analysed genes is in contrast to our previous study where we observed a strong host inflammatory and immune response to the parasite at the mRNA level following a single exposure (Pennacchi et al., 2014). This marked difference between our two studies may be due to the time post infection, since the fish in our first experiment were sampled 10 days post-exposure (Pennacchi et al., 2014) whereas in this trial collected 23 days post-exposure. In support of this two previous studies reported that AGD-lesions become fortified with mucosal epithelium as the infection progressed whereas at earlier infection the central venous sinus, connective tissues and AGD-lesions were infiltrated with inflammatory cells (e.g. granulocytes and macrophages) (Adams and Nowak, 2003; 2004). Secondly, the difference in the gene expression may be due to the different exposure concentration that was much higher in the previous study (2000 cells/L) causing a stronger hyperplastic response and inflammation in the affected gills (Pennacchi et al., 2014). Furthermore, even if the naïve fish were transferred from the control tank to the infection tank following a FW bath of the reinfected fish, there is the possibility that the amoebae surviving upon the gills of the FW bathed fish would disseminate and reinfect the naïve fish via cohabitation similarly to a field reinfection. In this scenario the naïve fish would have been exposed to the amoebae for almost 60 days. Nevertheless, a gill check at around 40 days showed no gross signs of infection.

A coordinated down-regulation of the inflammatory and immune response was suggested as the parasitic protozoan strategy to withstand or evade the host response (Morrison et al., 2006a; Sacks and Sher, 2002; Wynne et al., 2008a). A recent study regarding the involvement of Th pathways in AGD suggested that either an immune evasion strategy or an allergic mechanism may be caused by *N. perurans* in AGD-affected fish (Benedicenti et al., 2015). However, we did not observe a strong

coordinated decrease of the inflammatory and immune response following exposure to *N. perurans* most likely due to the differences in the experimental design.

In this study the gross gill score and image analysis methods used to assess the AGD severity showed good agreement, although the gross gill score was less reliable in cases of light AGD severity as previously reported (Clark and Nowak, 1999; Zilberg et al., 2001). In fact small AGD-lesions were difficult to detect during visual examination of the gills and were easier to visualise post-fixation. As a consequence the biopsy sampling technique used in this study did not allow to target small AGD-lesions (smaller than 10 lamellae). In support of this, a previous study showed that AGD-lesions affecting less than approximately 10 lamellae were undetectable during gross examination of the gills (Adams et al., 2004). Moreover, another reinfection trial showed that fish categorised as clear during the gill score had small lesions at histological examination (Wynne et al., 2008b).

In conclusion, this study contributed to our knowledge of AGD and in particular on the effects of reinfection on the host immune response. Overall, our results indicated little change in the inflammatory and immune response at the mRNA level in the gills of Atlantic salmon following reinfection and long-term infection with *N. perurans*. Given the different methodologies employed over the years by gene expression studies on AGD and considered the great cellular heterogeneity of gills compared to other organs contrasts between studies (including this) are inevitable. Therefore, we suggest that more methodological details should be provided in future studies on AGD allowing easier interpretation and comparison of the results. Especially, details on sample location, sample composition (e.g. lesion percentage, type of lesion, types and ratio of cell populations), fish histories, fish source/controls, replication, amoebae source (e.g. wild or cultured), challenge conditions and intensity, and timing of sample selection relative to disease progress should be provided. Understanding the mechanisms underlying AGD will contribute to the potential development of immunoprophylactic treatments or other practical solutions.

CHAPTER 4

IMMUNE REACTIVITY IN EARLY LIFE STAGES OF SEA-CAGE CULTURED PACIFIC BLUEFIN TUNA NATURALLY INFECTED WITH BLOOD FLUKES FROM GENUS CARDICOLA

Pennacchi, Y., Shirakashi, S., Nowak, B.F., Bridle, A.R. (2016). Immune reactivity in early life stages of sea-cage cultured pacific bluefin tuna naturally infected with blood flukes from genus *Cardicola*. Fish and Shellfish Immunology. In Press, Accepted Manuscript.

4.1. Abstract

Pacific bluefin tuna (PBT), *Thunnus orientalis*, due to its high average price on the market is an economically valuable fish species. Infections by blood flukes from the genus *Cardicola* (Digenea: Aporocotylidae) represent a growing concern for the cage culture of bluefin tuna in Japan, Australia and Southern Europe. The accumulation of numerous *Cardicola* eggs in the fish gills causes severe pathology that has been linked to mortality in PBT juveniles up to one year old. The only effective treatment used to mitigate the infection is the oral administration of the antihelminthic drug praziquantel (PZQ) to the affected fish. However, with the need to minimise therapeutic drug use in aquaculture it is hoped that immunoprophylaxis can provide a future alternative to protect the PBT juveniles against *Cardicola* infection. Currently, little is known of the host immune response to these parasites and of their infection dynamics. In this study, using qPCR we aimed to quantitatively detect *C. orientalis* and *C. opisthorchis* DNA within the gills and heart of cultured PBT juveniles and to investigate the host immune response at the transcriptional level in the gills. The research focused mainly during early stages of infection soon after young PBT were transferred to culture cages (from 14 to 77 days post-transfer). An increased (up to 11-fold) transcript expression of immune-related genes, namely IgM, MHC-I, TCR-β and IL-1β was observed in the PBT gills infected with *Cardicola* spp. (28-77 days post-transfer). Furthermore, IgM (19-fold increase) and MHC-I (11.5-fold increase) transcription was strongly up-regulated in gill samples of PBT infected with *C. orientalis* relative to uninfected fish but not in fish infected with *C. opisthorchis*. *Cardicola*-specific DNA was first detected in the host 14 days post-transfer (DPT) to sea-cages which was 55 days earlier than the first detection of parasite eggs and adults by microscopy. Oral administration of PZQ did not have an immediate effect on parasite DNA presence in the host and the DNA presence started to reduce after 24 days only in the host heart. The results provide evidence of an immune response in early age sea-cage cultured juveniles of PBT naturally infected with *C. orientalis* and *C. opisthorchis*. This

response, whilst not protective against primary infection, provides evidence that immunisation at an early age may have potential as a health strategy.

4.2. Introduction

The Pacific bluefin tuna (PBT) (*Thunnus orientalis*) is one of the most popular tuna species and it has a high economic value. As with other commercially relevant bluefin tuna species PBT aquaculture is mainly based on a capture and grow-out approach since its farming relies on the stocking of wild-caught individuals. In 2002, after 32 years of study, researchers at Kindai University, Japan, closed the PBT life cycle in captivity to face the increasing demand for PBT in the Japanese market and to minimise the exploitation of wild stock (Sawada et al., 2005). Continued improvements in rearing techniques have allowed increased production and in 2012 the Japanese PBT production was 9,592 metric tons, of which 244 metric tons were derived from hatchery-reared juveniles (Benetti et al., 2016). One of the most critical phases during PBT farming is the transfer of fingerlings (approximately 30 days old) from land-based facilities to sea cages (Miyashita, 2002).

Following the transfer to sea cages, fish are exposed for the first time to different pathogens of which two blood fluke species ascribed to the genus *Cardicola* (Trematoda: Aporocotylidae), *C. orientalis* and *C. opisthorchis*, represent the greatest concern for the Japanese tuna farming industry (Ogawa, 2015). *C. orientalis* is known to infect mainly the gill arteries whereas *C. opisthorchis* is predominant in the heart (Ogawa et al., 2010; Ogawa et al., 2011). Co-infections by *C. orientalis* and *C. opisthorchis* are common, however the eggs of these two species are distinguishable as *C. orientalis* have smaller-ovoid shaped eggs while *C. opisthorchis* eggs are a larger-crescent shape (Shirakashi et al., 2012b). Eggs can accumulate in the gills and eventually cause the fish to die of suffocation (Shirakashi et al., 2012a). Juvenile PBT up to one year old are the most threatened by *Cardicola* spp. and to mitigate the recurrent infection outbreaks an anthelmintic drug, praziquantel (PZQ), is orally administered to fish (Shirakashi et al., 2012a). Immunomodulatory abilities and the

potential capacity to act as an immune stimulant/adjuvant have been attributed to PZQ (Polinski et al., 2014a). Recently, a third *Cardicola* species, *C. forsteri*, was reported in farmed PBT in Japan (Shirakashi et al., 2016). *Cardicola* spp. represent a threat not only to the PBT farming in Japan but also to the cage culture of other bluefin tuna species worldwide (Ogawa et al., 2010; Ogawa et al., 2011; Palacios-Abella et al., 2015; Shirakashi et al., 2013). In the Mediterranean, four species of *Cardicola* (*C. forsteri*, *C. orientalis*, *C. opisthorchis* and a not yet described species) are known to infect Atlantic bluefin tuna (ABT) (*Thunnus thynnus*) cage-reared adult individuals (Palacios-Abella et al., 2015) and infection outbreaks by *C. forsteri* and *C. orientalis* represent a recurrent issue for the cage-culture of the Southern bluefin tuna (SBT) (*Thunnus maccoyii*) in Australia (Aiken et al., 2009; Cribb et al., 2011; Polinski et al., 2013c).

Until recently, the only method to determine a *Cardicola* spp. infection in bluefin tuna was through the use of gill microscopy and heart flushes, respectively to identify the parasite's eggs and adults. A recent study showed the usefulness of qPCR techniques as a diagnostic method to differentially and quantitatively identify the presence of *Cardicola* spp. DNA in bluefin tuna organs (Polinski et al., 2013a). Furthermore, compared to microscopy, the qPCR diagnostic method allows the farmers to determine the onset of infection at earlier developmental stages (Polinski et al., 2013a; Polinski et al., 2013c; Polinski et al., 2014b). In fact, the qPCR is able to detect (but not distinguish) the DNA of any *Cardicola* life cycle stage (Polinski et al., 2013c).

In the last decade, the parasitic infections of blood flukes belonging to the genus *Cardicola* became of worldwide interest due to the high commercial value of the fish host. Although extensive research has been carried out on the bluefin tuna, little is known about the mechanisms involved in the host immune response to *Cardicola* spp. Nevertheless, there is evidence of a serum antibody response in SBT (Aiken et al., 2008) and resistance to recurrent infection during the first year of cage culture in PBT (Shirakashi et al., 2012a), which suggest a partially protective anti-*Cardicola* spp. antibody response is developed. Recently, investigations of the PBT immune response to *Cardicola* spp. infection have shown an increased transcription of selected immune genes in the gill (IgM) and

heart (IgM, MHC-II, TCR-β, and IL8) of infected juveniles relative to non-infected fish during long-term infection (Polinski et al., 2014b). A highly significant positive correlation between the relative quantity of IgM transcription and *C. orientalis* DNA relative abundance in the gills was shown for the same fish (Polinski et al., 2014b). Altogether these previous results on the PBT immunity against *Cardicola* suggest that host immunisation may represent a future possible prophylactic health strategy.

The aims of this study were to investigate the PBT early life stages immune response to *Cardicola* spp. infection in the main affected organ – the gills, determine the relative prevalence of *C. orientalis* and *C. opisthorchis* in PBT gill and heart and investigate the relationship between the immune response and the relative prevalence of each *Cardicola* spp. To achieve these aims, qPCR techniques were used to differentially detect *Cardicola* spp. DNA in the host gills and heart and to investigate the host immune gene expression at the mRNA level. Furthermore, routine histology was used to describe the pathology associated with *Cardicola* spp. eggs in the host gills. The current study was focused on early rearing of PBT, which is the farming stage with the highest recorded mortality during cage culture due to *Cardicola* spp. infection. Given the recent finding of a third *Cardicola* species (*C. forsteri*) found to infect PBT, we also aimed to describe its presence and relative prevalence.

4.3. Materials and methods

4.3.1. Juvenile PBT farm history

The use of juvenile PBT and the experimental procedures were approved by the University of Tasmania (Animal Ethics Committee Permit No. A0013248). PBT came from a broodstock of farm raised fish at Oshima Station, Aquaculture Research Institute, Kindai University, Wakayama prefecture, Japan. The spawning occurred around the 19^{th}-20^{th} of June 2013 and fertilised eggs were collected from sea cages and transferred to Oshima land based facilities where the hatching occurred

on the 21st of June. On the 24th of July, fish were transferred into sea cages. Fish were fed a commercial diet and frozen sand lance (*Ammodytes* spp.) to satiation throughout the duration of the study. Mortality in the sea cages was checked and recorded daily and any dead fish were removed by technical personnel to prevent the spread of diseases. In the individuals used in this study, infection by *Cardicola* spp. eggs and adults was first detected by microscopy in the gills and heart at 69 days post transfer (DPT) to sea cages. The antihelminthic praziquantel (PZQ) (150 mg/kg) was orally administered to fish 50-53 DPT after first detection of the *Cardicola* spp. infection in the PBT juveniles stocked in nearby cage facilities.

4.3.2. Sampling strategy and samples collection

Juvenile PBT (n = 45), five fish each sampling, were collected at 14, 21, 28, 35, 42, 49, 56, 69 and 77 DPT from the same sea cage. At the first sampling (14 DPT) the fish average weight was 1.2 g and length 4.2 cm and at the last sampling (77 DPT) the average weight was 233.3 g and length 20.9 cm. Fish were caught from the cage using a small dip net, immediately killed by cranial concussion following standard industrial practice, and put on ice until dissection. For histological analysis the second left gill arch was dissected, rinsed with filtered seawater and immediately fixed in 10% neutral buffered formalin. After 24 hours, histological samples were transferred to 70% ethanol. For molecular analysis the second right gill arch and the ventricle of the heart were dissected, rinsed with filtered seawater and immediately placed in an RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2). The samples collected in RNA preservation reagent were temporarily stored at 4^0C overnight and then at -20^0C for long term storage. Following each sampling 5-10 gill filaments from the first gill arch of each fish were placed on a slide and examined under a light microscope for the presence of *Cardicola* spp. eggs and the heart checked for the presence of *Cardicola* spp. adults using a dissecting microscope.

4.3.3. Gill histology

Samples were decalcified for 45 min using the Rapid decalcifying fluid (Australian Biostain Pty. Ltd.) then processed for routine histology in a Leica TP1050 tissue processor by being dehydrated in a sequence of increasing concentration of ethanol (80%, 95% and 100%), then immersed in xylene and finally in paraffin. Samples were embedded in clean paraffin, sectioned (5 μm) and stained with Hematoxylin and Eosin (H&E).

4.3.4. Cardicola spp. infection assessment in the gills

Gill sections were scanned at 400 x magnification under a compound light microscope Olympus BH-2 (Olympus, Hamburg, Germany) to confirm the presence of *Cardicola* spp. eggs, assess the severity and to describe the pathology associated with the presence of *Cardicola* spp. eggs (**Table 4.1.**). Gill sections were photographed using a Leica DC 300F digital camera (Leica Microsystems Inc., Illinois, USA). The total number of filaments was counted for each gill section and filaments were counted only when the central venous sinus (CVS) was visible in at least two-third of its length in each gill filament as described by Adams and Nowak, 2004. Based on their shape the eggs having an oval shape were recorded as *C. orientalis* whereas eggs with a crescent shape were identified as *C. opisthorchis* (see Shirakashi et al., 2012b). *Cardicola* infection severity was assessed using the following method: filaments containing at least one egg of either species of *Cardicola* were counted as affected filaments and then the percentage of affected filaments was calculated using the following equation:

$$\text{Percentage of affected filaments} = 100 \text{ x } \frac{\text{N affected filaments}}{\text{N total filaments}}$$

Infection level	N affected filaments	Histopathology
Clear	0	No eggs or any sign of pathology associated with *Cardicola*
Light	1-19	A few eggs, localised lamellar fusion, a few granulomas
Moderate	20-40	Numerous eggs, lamellar fusion covering up to half of the gill arch area, swollen filament arteries, granulomas
Heavy	> 40	Numerous eggs, extensive lamellar fusion covering more than half of the gill arch area, swollen filament arteries, widespread granulomas

Table 4.1. *Cardicola* spp. infection assessment used during histology analysis to assess the infection level of 2nd left gill arch.

4.3.5. RNA extraction and cDNA synthesis from PBT gill

The total RNA was extracted from 10 mg of RNA preservation reagent stabilised gill samples (14, 28, 35, 42, 49, 56, 69 and 77 DPT) by using 350 µL of Modified UNEX extraction buffer (2.25M GITC, 0.2% Tx100, 0.14M NaCl, 0.07M NaOAc, 0.01% SDS, pH6) containing 5 µL of Proteinase K and 2 µL of 1M DTT (Dithiothreitol) per sample and homogenising immediately using a micro tube-pestle (excess of RNA preservation reagent was removed by blotting samples). Cellular debris was removed by centrifuging at 12,000 xg for 5 min. Supernatants were transferred to fresh tubes adding an equal volume of isopropanol to recover nucleic acids, washed 1x with 70% ethanol and resuspended in 100 µL 1x DNAse buffer (10 mM Tris, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM DTT) at 37 ºC. Samples were treated with 4U of DNAse (Ambion® TURBO™ DNase) for 1 hr at 37 ºC. After the DNAse treatment 900 µL of GITC buffer (4M GITC, 1% Tx100, 50 mM Tris, pH7) with 500 µL of 100% ethanol was added to each sample. Samples were transferred to silica spin columns placed in 2 mL microfuge tubes and centrifuged. This last step was repeated until all sample passed over the column. Then, to wash the columns 700 µL of RW1 (Qiagen) (1x) and 500 µL of RPE (Qiagen) (2x) were added, centrifuged and flow discarded through. To elute RNA, the columns were

transferred to new 1.5 mL tubes adding 90 µL of RNAase free water twice and keeping flow through. After discarding columns, 20 µL 10x DNAse buffer with 1 µL DTT were added to each eluted 180 µL sample. A second DNAse treatment with 4U of DNAse was performed followed by a purification step. Ultimately, the DNA-free total RNA was re-suspended in 50 µL RNA resuspension buffer (1mM sodium citrate, 5mM DTT), quantified using a Qubit fluorometer (Invitrogen) and an aliquot run on a 1% Agarose gel stained with GelRed to verify the integrity of the total RNA. All extracted RNA samples had a well-defined 28S and 18S rRNA bands with the 28S twice as intense as the 18S band showing no sign of degradation. After RNA extraction a cDNA Synthesis Kit (Bioline) with Oligo (dT)18 primer mix was used, following manufacturer instructions, to reverse transcribe 1 µg of total RNA into cDNA. Controls, lacking reverse transcriptase, were run to confirm that genomic DNA contamination did not occur.

4.3.6. Isolation of C. orientalis and C. opisthorchis DNA from PBT gill and heart

Total nucleic acid (TNA) was extracted from each gill (average weight 148 mg) and heart (average weight 14 mg) sample (14, 21, 28, 35, 42, 49, 56, 69 and 77 DPT) by digesting and lysing the cells in 2 mL Urea extraction buffer (4 M Urea, 1% SDS, 0.2 M NaCl, 1 mM Na citrate) supplemented with 6 µL Proteinase K (Bioline). Samples were incubated overnight at 37 ºC (until fully digested) and then 500 µL were processed whereas the leftover samples (1.5 mL each) were frozen at -20 ºC. TNA in the 500 µL samples was selectively separated from cellular debris and protein by adding 300 µL 7.5 M ammonium acetate and centrifuging samples at 18 ºC, 14,000 xg, for 5 min. Supernatants were decanted to clean tubes adding an equal volume of isopropanol with co-pink precipitate (Bioline) to recover nucleic acids. These last two steps (separation from cellular debris/protein and recover of nucleic acids) were repeated for gill samples to completely remove all cellular debris. Samples were resuspended in 100 µL 10 mM Tris-buffered water (pH 7-8) supplemented with 0.05% TX 100.

4.3.7. Quantitative real-time PCR and data analysis

A CFX Connect Real-Time PCR detection system (Bio-Rad, NSW, Australia) was used to conduct all qPCR analyses. All the targeted genes and primer sequences used in this study are listed in **Table 4.2.**. Following a previously described method (Polinski et al., 2013c) the internal transcribed spacer-2 (ITS2) region of rDNA specific to *C. orientalis, C. opisthorchis* and *C. forsteri* was used to carry out targeted quantitative PCR amplification for PBT gill and heart. Each PCR reaction consisted of 2X My Taq HS Mix, forward and reverse primers (500 nM each), 100 nM TaqMan HEX-labelled probe and 2 µL gill and heart-derived TNA template in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicates alongside a six-step ten-fold dilution series of the samples and cycling conditions consisted of an initial activation of DNA polymerase at 95 °C for 3 min, followed by 45 cycles of 10 s at 95 °C, and 30 s at 60 °C. No-template controls were run. Limit of Quantification (LOQ) with a 95% confidence level was 8 copies when quantifying *C. forsteri*, 16 copies for *C. orientalis* and 25 copies for *C. opisthorchis* (Neumann et al., Unpublished). The limit of detection (LOD), where the assay accurately detected expected ITS2 rDNA numbers was 4 copies for *C. forsteri* and *C. opisthorchis*, and 8 copies for *C. orientalis* (Neumann et al., Unpublished).

The gene expression of the inflammatory and immune related genes IL-1β, IL-8, IgM(H), TCR-β, MHC-I and MHC-II was carried out using the cDNA described above. SYBR green chemistry was used to measure the differential expression of the target genes. PCR reactions were prepared in 2X My Taq HS Mix + SYBR, forward and reverse primers (400 nM each), and 2 µL cDNA template (1 µg RNA) in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicate and cycling conditions consisted of an initial activation of DNA polymerase at 95 °C for 3 min, followed by 39 cycles of 10 s at 95 °C, 20 s at 60 °C, and 10 s at 72 °C. No-template and no-reverse transcription controls were run. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity with fluorescent detection conducted at 0.5 °C intervals.

qPCR runs were only considered for analyses when amplification efficiency (E) of standard dilutions ranged between 85-105% with a linear regression (r2) of 0.98-1.00.

4.3.8. Statistical analysis

The Bioline CFX interface software was used to estimate the genomic copies for both *Cardicola* spp. Gene expression was analysed with qBasePLUS® analysis software (Biogazelle, Zwijnaarde, Belgium) by copying the cycle threshold (Ct) values to an excel spread sheet and importing data into qBasePLUS to carry out the qPCR data analysis as previously described (Hellemans et al., 2007). The mean expressions of the two reference genes elongation factor 1-alpha (EF1-α) and beta-actin (β-actin) (**Table 4.2.** for primer sequences) was used to normalise the results (Vandesompele et al., 2002), which were scaled to the minimum value. The qPCR data were analysed with qBase plus software as described by (Hellemans et al., 2007) where mRNA expression was normalised using the geometric mean of expressions of the two reference genes ($M<1$; $CV<0.5$) (Polinski et al., 2013b; Vandesompele et al., 2002). To determine differences in the immune gene mRNA expression among the different groups a one-way ANOVA corrected for multiple measures was run. To assess the effects of infection by *Cardicola* spp. on the fold induction of each immune gene a Mann-Whitney one-tailed U-test was used. A 2-fold induction or repression was considered as minimum level of biological significance. Additionally, Pearson's correlation analysis was used to compare the mean normalised relative quantities of selected genes to relative quantities of *C. orientalis* and/or *C. opisthorchis* DNA. Values were considered statistically different at *P*-values < 0.05.

Name	Target	Accession	Product size	Sequence 5' → 3'
tuna_q_actin_F	β-actin	JX157141	89 bp	TATCCTGACCCTGAAGTA
tuna_q_actin_R				CATTGTAGAAGGTGTGATG
tuna_q_EF1a_F	EF1-α	JX157143	86 bp	TTGGTGTCAACAAGATGG
tuna_q_EF1a_R				GATGTAGGTGCTCACTTC
tuna_q_IL1_F	IL-1β	JX157146	92 bp	AGCCACAAGATAACCAAG
tuna_q_IL1_R				TTCTCTACAGCGATGATG
tuna_q_IL8_F	IL8	JX157147	108 bp	CTACTGTTCGCTTGTCGCTAA
tuna_q_IL8_R				TTGATAGGTTGTCATCGGACTTAC
tuna_q_IgM_F	IgM (H)	EC917943	113 bp	GTTCCACTTGACATCACAT
tuna_q_IgM_R				AGTCCTACAGTCCTCCTT
tuna_q_MHC2_F	MHC-II	EH667387	344 bp	CAAGTTGGAGCACATCAG
tuna_q_MHC2_R				ATCTCATCGGTGGAAGTG
tuna_q_TCR_F	TCR-β	EC092872	101 bp	AAGAGCAGCGTCTATGGA
tuna_q_TCR_R				CTTGAGAGTTCACTTGTTCAG
tuna_q_MHC1_F	MHC-I	EH667956	551 bp	TGATGTACGGCTGTGAAT
tuna_q_MHC1_R				TGTCTGACCTCTGTTATGAT
Cori_F	*C.orientalis*	HQ324226	191 bp	TGCTTGCTATTCCTAGATGTTTAC
Cori_R	(ITS2 rDNA)			AACAACTATACTAAGCCACAA
Cori_probe				HEX – CACAAGCCGCTACCACAATTCCACTC – BHQ1
Copis_F	*C.opisthorchis*	HQ324228	272 bp	TTCCTAAATGTGTGTGCA
Copis_R	(ITS2 rDNA)			TCAAAACATCAATCGACACT
Copis_probe				HEX – CACGACCTGAGCACAAGCCG – BHQ1
Cfor_F	*C.forsteri*	EF661575	287 bp	TGATTGCTTGCTTTTTCTCGAT
Cfor_R	(ITS2 rDNA)			TATCAAAACATCAATCGACATC
Cfor_probe				HEX – CCACGACCTGAGCACAAGCCG – BHQ1

Table 4.2. Specific primers and probes used for PBT immune gene expression and *Cardicola* detection.

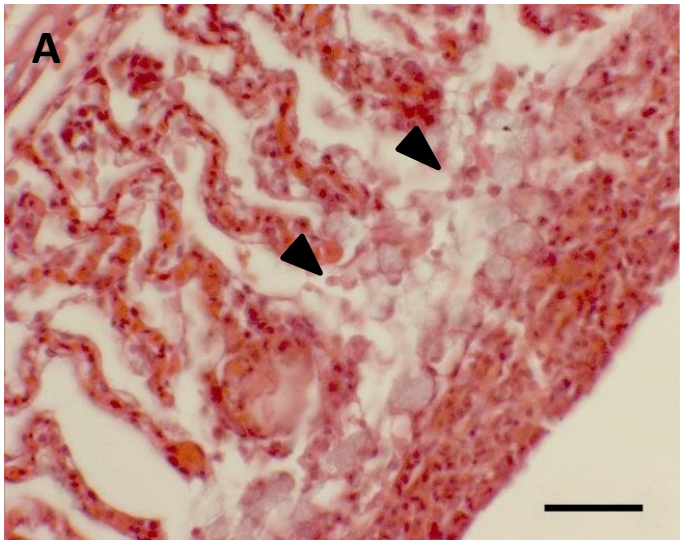
4.4. Results

4.4.1. Gill and heart microscopy

We first observed *Cardicola* spp. adults in the heart and eggs in the gills at 69 DPT. Nevertheless, routine samplings carried out in the nearby juvenile PBT sea cages showed that *Cardicola* spp. adults and eggs first appeared at 49 DPT.

4.4.2. Gill histopathology

Cardicola spp. eggs were observed only in the gill sections at 69 DPT. Two out of five fish showed light signs of infection by *Cardicola* spp. (percentage of affected filaments ± SD: 4.3 ± 10.7). A few oval shaped *C. orientalis* eggs wrapped by layers of fibroblasts at lamellae tips (**Fig. 4.1.: B**) with occasional lamellar fusion were observed in gill filaments. Granulomas mainly associated with different developmental stages of *C. opisthorchis* eggs and close to filament arteries were detected. Occasionally epithelial hyperplasia with numerous mucous cells distributed mainly between the apical part of the lamellae and the hyperplastic structure was observed in the distal areas of the gill arch (**Fig. 4.1.: A**). *C. orientalis* eggs were present mainly within the middle or distal area of the gill arch at lamellae tips and occasionally close to filament arteries, whereas crescent shaped eggs ascribed to *C. opisthorchis* were mainly detected in distal area of the gill arch and in or close to filament arteries. *Cardicola* spp. eggs were not visualised in gill sections of other time points.



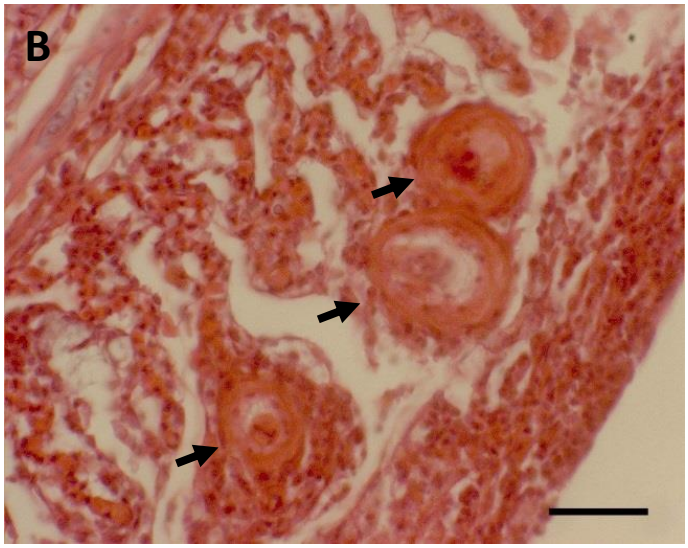


Fig. 4.1. H&E stained 5 μm histological sections of juvenile PBT gills at 69 DPT. In A several mucous cells (arrowheads) are between the lamellae and hyperplastic epithelia. In B different developmental stages of *C. orientalis* eggs (arrows) are present in the lamellae tips (bar = 50 μm).

4.4.3. Detection of Cardicola spp. in cultured juveniles PBT gills and heart

In the gills *C. orientalis* DNA was first detected by qPCR in samples collected at 21 DPT whereas *C. opisthorchis* DNA was first detected at 35 DPT (**Fig. 4.2., 4.3.: A**), while gill microscopy and histology first identified *Cardicola* spp. eggs at 69 DPT. In PBT heart samples *C. orientalis* DNA was first detected at 69 DPT whereas *C. opisthorchis* DNA was first present in heart samples collected at 14 DPT (**Fig. 4.2., 4.3.: B**). Similarly to the detection of eggs using gill microscopy, *Cardicola* spp. adults were first found in heart flushes at 69 DPT. The highest relative level for both *Cardicola* spp. DNA was observed in heart samples at 69 DPT, in particular *C. opisthorchis* DNA reached approximately $7x10^{12}$ copies/mg and *C. orientalis* DNA $2x10^{12}$ copies/mg (**Fig. 4.3.: B**). In the gills *C. opisthorchis* DNA had the highest relative level (approximately $1x10^{6}$ copies/mg) at 69 DPT, and *C. orientalis* DNA at 21 DPT with a similar number of copies/mg ($1x10^{6}$) (**Fig. 4.3.: A**). In general, the gill became infected rapidly and to a greater extent by *C. orientalis* and both *Cardicola* spp. showed a fluctuating trend during the infection progression (**Fig. 4.2., 4.3.: A**).

In the heart the onset of infection, started by *C. opisthorchis,* was earlier compared to the gill with the same species markedly predominant during the cage culture (**Fig. 4.2., 4.3.: B**). In both examined organs, the administration of PZQ treatment did not show an immediate effect on the relative quantity of both *Cardicola* spp. DNA. In the heart, 16 days post PZQ treatment both *Cardicola* spp. DNA reached the highest relative level that decreased to zero at 24 days post treatment (**Fig. 4.3.: B**). The *Cardicola* spp. approximate mean genomic copies/mg of PBT organ detected by qPCR during cage culture are summarised in **Table 4.3.**. Furthermore, *C. forsteri* DNA was not detected in any of the PBT gill and heart samples analysed.

4.4.4. Immune gene expression in PBT gill infected by Cardicola spp.

Four (IgM, MHC-I, TCR-β, and IL-1β) out of six (IgM, MHC-I, TCR-β, IL-1β, IL8, and MHC-II) immune-related genes analysed were significantly induced ($P < 0.05$) in juvenile PBT gill infected by *Cardicola* spp. during sea-cage culture (**Fig. 4.4.**). IgM showed a marked increase (8-11-fold increase) of the mRNA expression during late time points relative to early time points ($P < 0.05$) (**Fig. 4.4.: A**). Similarly to IgM, MHC-I mRNA expression was strongly up regulated (4-11-fold increase) in late time points relative to early time points ($P < 0.05$) (**Fig. 4.4.: B**). An increase (range 2-3-fold increase) of TCR-β mRNA expression was observed at 49, 69 and 77 DPT relative to 14 DPT ($P < 0.05$) (**Fig. 4.4.: C**). The pro-inflammatory cytokine IL-1β showed a slight transcriptional increase (approximately 2-fold) at 28 DPT relative to 35 DPT ($P < 0.05$) **(Fig. 4.4.: D**). Following the PZQ treatment (50-53 DPT) only MHC-I mRNA expression showed a marked decrease whereas the other immune-related genes significantly induced during *Cardicola* spp. infection had a modest decrease and increased again at 77 DPT (**Fig. 4.4.**).

The effects of *C. orientalis* and *C. opisthorchis* infection on gene expression were assessed for each gene. MHC-I (11.5-fold) and IgM (19-fold) mRNA expression was up-regulated ($P < 0.05$) in gill samples positive (n=11) for *C. orientalis* relative to negative samples (n = 27) (**Fig. 4.5.**). Additionally, MHC-I was the only up-regulated gene (7.7-fold) ($P < 0.05$) in the infected gills (n =

20) (considering both *Cardicola* spp.) relative to non-infected samples (*n* = 18) (**Fig. 4.6.**). None of the other immune-related genes analysed were significantly induced by the presence of *Cardicola* spp. (data not shown). For all gill samples the infectious status for both *Cardicola* spp. was assessed by qPCR. The correlation analysis between *Cardicola* spp. DNA quantity and relative transcriptional quantity for each gene analysed showed that IgM (r = 0.394) and MHC-I (r = 0.565) transcription in PBT gills was positively correlated to the relative quantity of *C. orientalis* DNA (**Fig. 4.7.**). For all the other genes analysed there were no significant correlations between *Cardicola* spp. DNA quantity and transcriptional relative quantity (data not shown). The expression of the reference genes (EF1-α and β-actin) remained constant and no significant change was detected.

DPT	**GILL**		**HEART**	
	C. orientalis	*C. opisthorchis*	*C. orientalis*	*C. opisthorchis*
14	0	0	0	$3x10^{11}$
21	$1x10^{6}$	0	0	0
28	$2x10^{4}$	0	0	0
35	0	$5x10^{5}$	0	10^{7}
42	$4x10^{5}$	$1x10^{4}$	0	10^{2}
49	$6x10^{4}$	$2x10^{4}$	0	0
56	$1x10^{4}$	$1x10^{4}$	0	$8x10^{7}$
69	$9x10^{3}$	$1x10^{6}$	$2x10^{12}$	$7x10^{12}$
77	$3x10^{5}$	$6x10^{4}$	0	0

Table 4.3. Relative level of *Cardicola* spp. in PBT gill and heart following the transfer to sea-cage. Data present the approximate mean genomic copies of *Cardicola* spp. ITS2 rDNA (copies/mg) detected in host organs by qPCR. DPT stands for days post transfer.

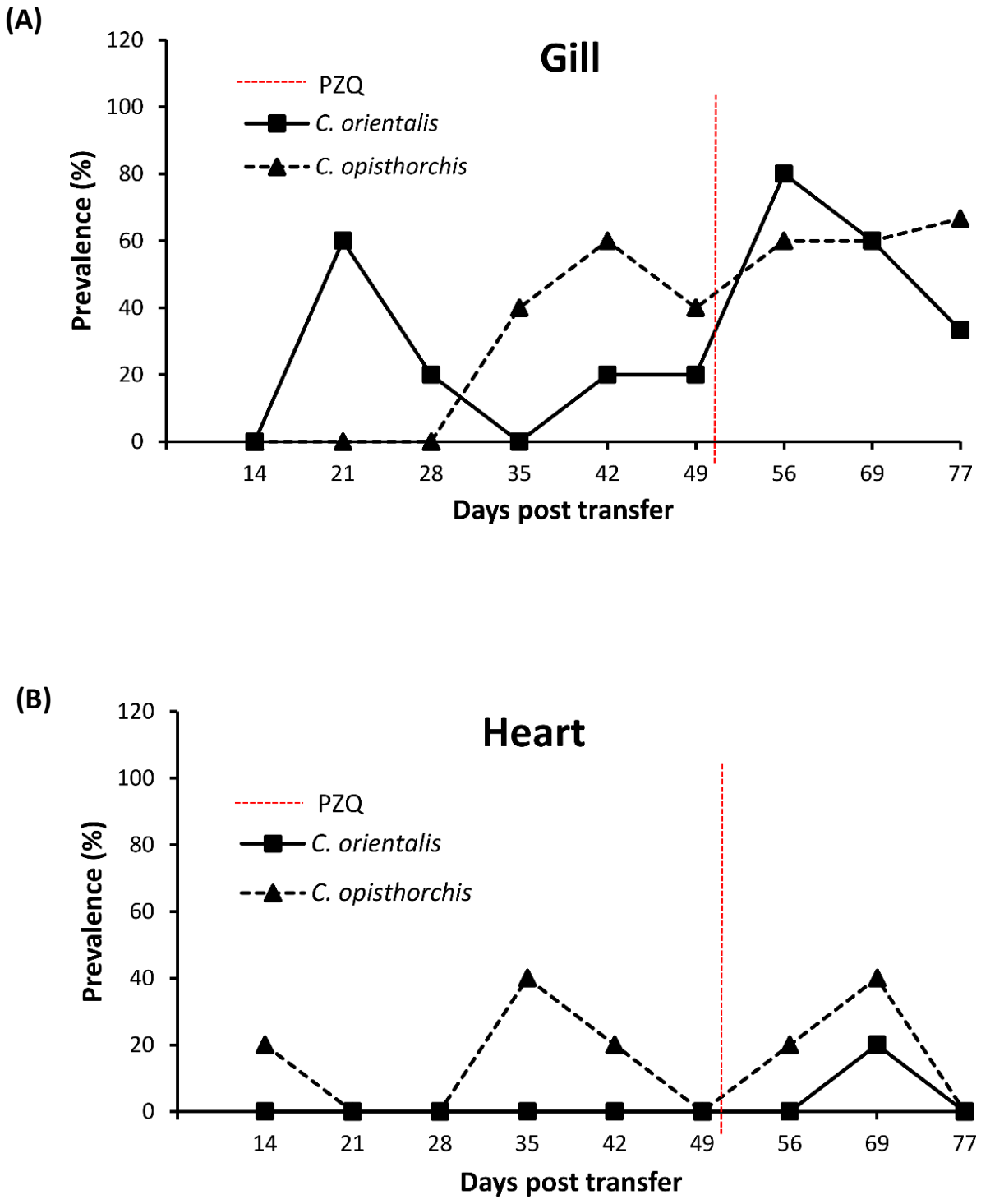


Fig. 4.2. Relative prevalence of *Cardicola* spp. in PBT gill and heart during sea-cage culture. Data indicate the proportion of infected individuals in the sample population at the time point indicated. The dashed line indicates timing of oral anthelmintic praziquantel (PZQ) treatment.

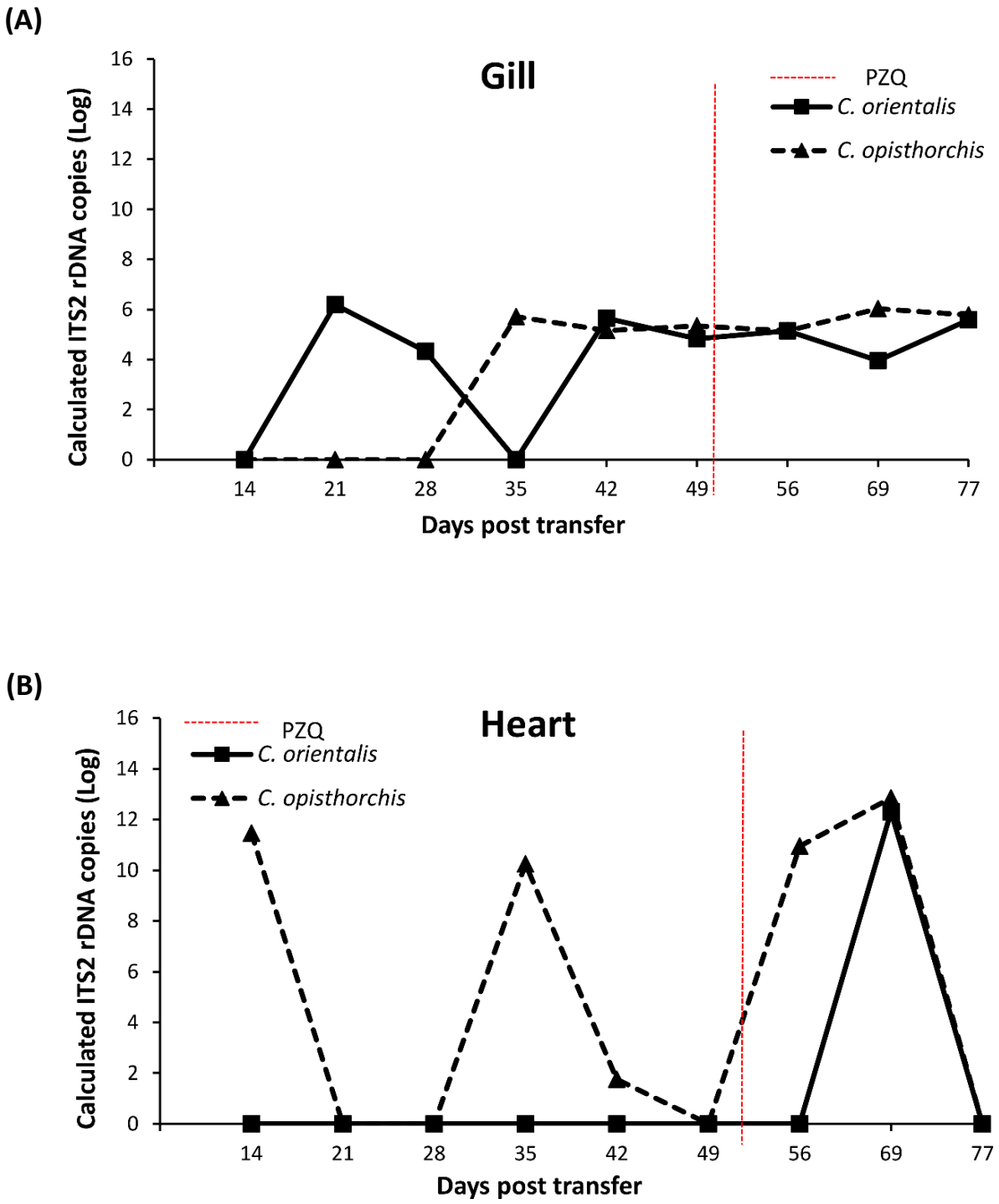
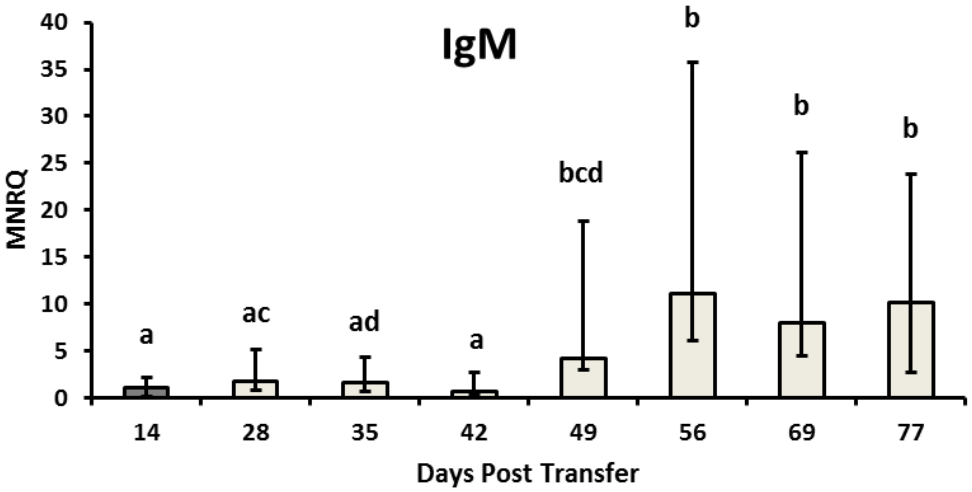


Fig. 4.3. Relative copy numbers of *Cardicola* spp. DNA in PBT gill and heart following the transfer to sea-cage. Data present the mean genomic copies of *Cardicola* spp. DNA detected in host tissues by qPCR. The dashed line indicates timing of oral anthelmintic praziquantel (PZQ) treatment.

(A)



(B)

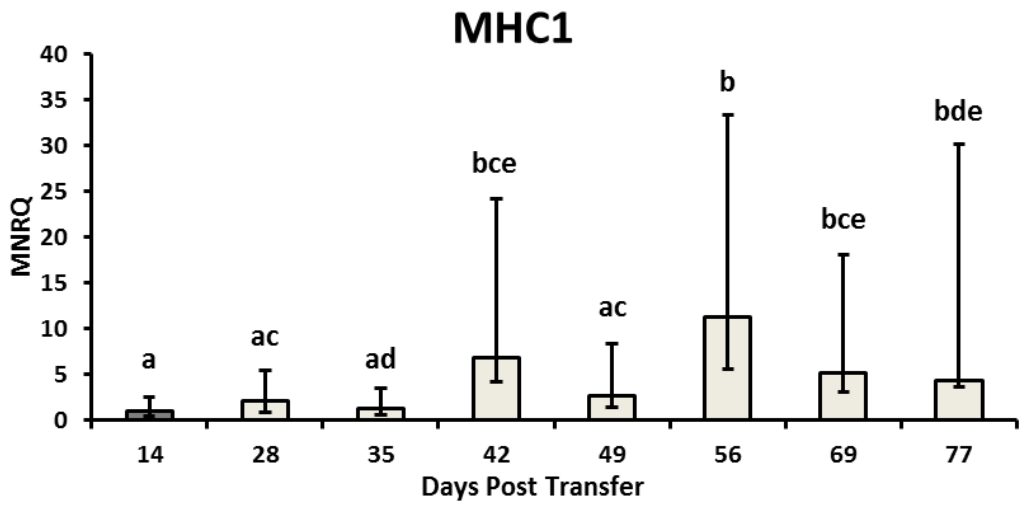
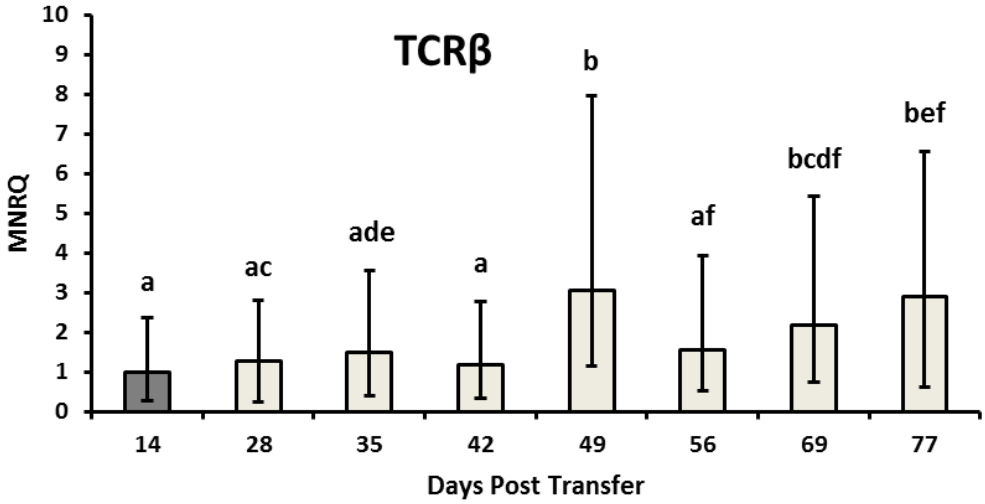


Fig. 4.4. The geometric mean normalised relative quantity (MNRQ) of immune related genes significantly expressed during *Cardicola* spp. infection in PBT gill samples in relation to days post transfer to sea-cage. The error bars represent the 95% confidence interval.

(C)



(D)

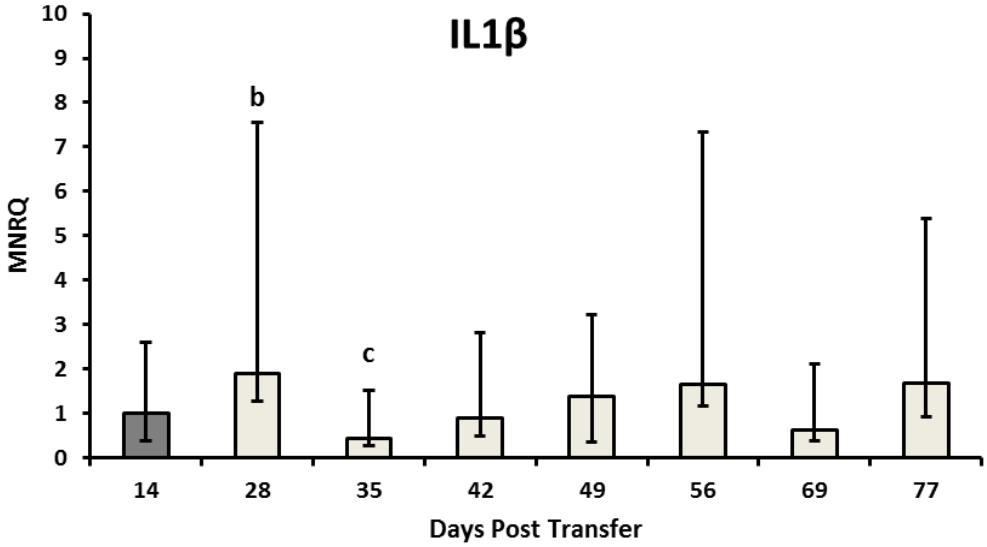


Figure 4.4. Continued.

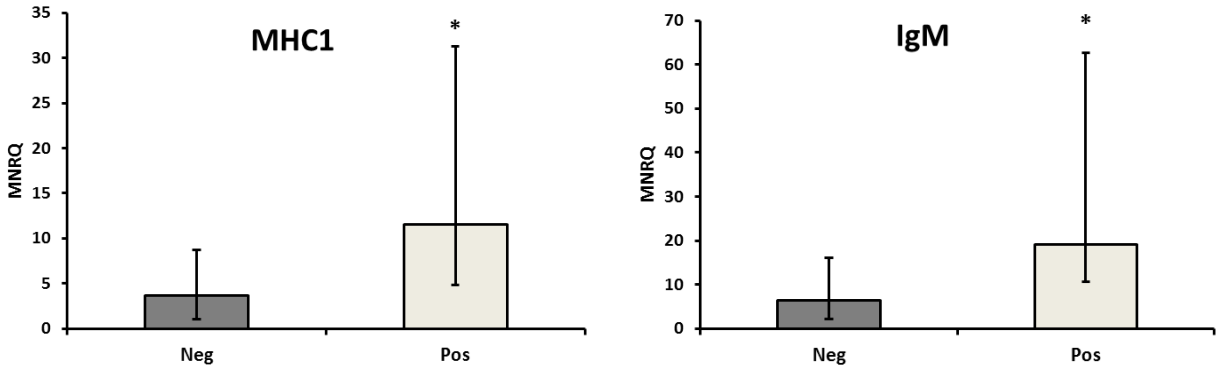


Fig. 4.6. The effects of *C. orientalis* infection on immune gene expression in the gill during PBT cage culture. The bars represent the 95% confidence interval. (*) Identifies a significant ($P < 0.05$) induction relative to non-infected fish. MNRQ stands for geometric mean normalised relative quantity.

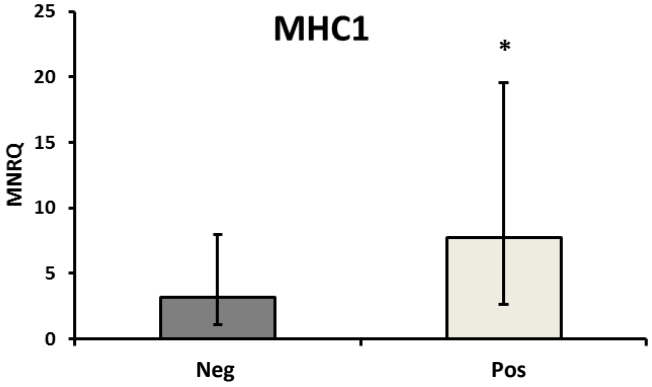


Fig. 4.5. The effects of *Cardicola* spp. infection on immune gene expression in the gill during PBT cage culture. The bars represent the 95% confidence interval. (*) Identifies a significant ($P < 0.05$) induction relative to non-infected fish. MNRQ stands for geometric mean normalised relative quantity.

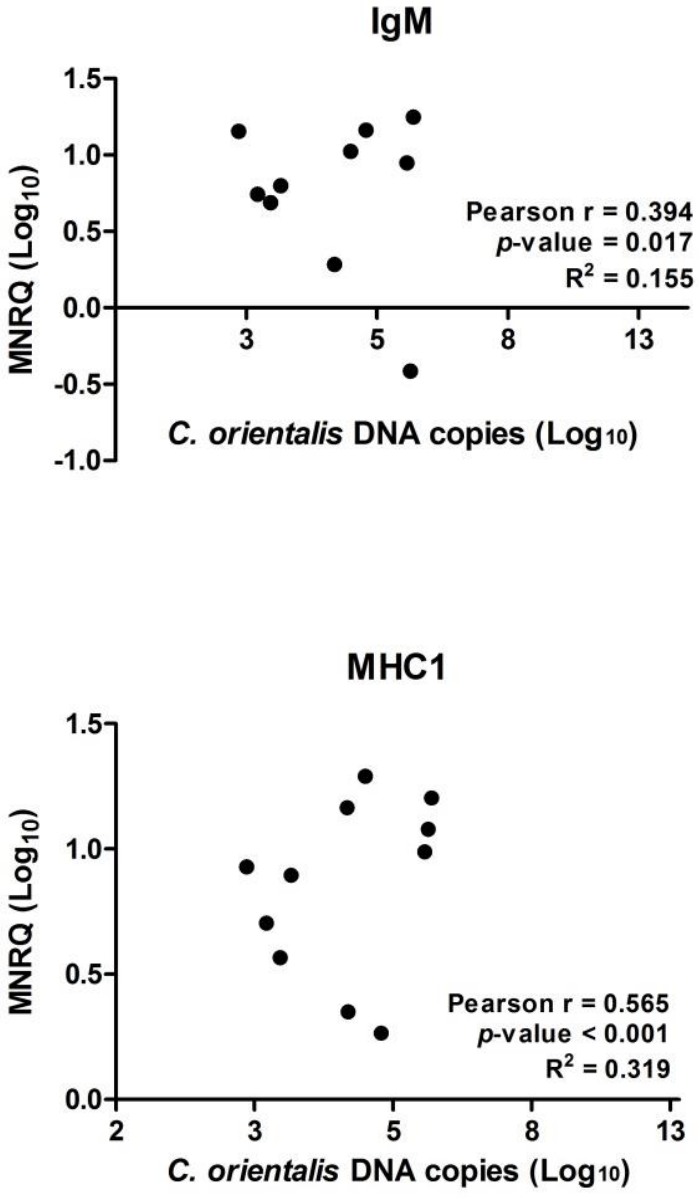


Fig. 4.7. Relationship between *C. orientalis* ITS rDNA quantity and IgM and MHC1 transcription in the gill of cage cultured PBT. MNRQ stands for geometric mean normalised relative quantity.

4.5. Discussion

Infections with *Cardicola* species represent a serious concern for the PBT mariculture in Japan as they have been linked to mortality of the affected individuals especially in juveniles up to one year old (Shirakashi et al., 2012b). To mitigate *Cardicola* infection outbreaks the PBT industry currently relies solely on the oral administration of the antihelminthic drug PZQ (Shirakashi et al., 2012a). Research on alternative solutions aimed to boost the PBT immune system is still scarce and as such investigations of the host immunity to the parasite are critical to further improve PBT health in mariculture. In this study the onset of *C. orientalis* and *C. opisthorchis* infections in early life stages of sea-cage cultured PBT was earlier than previously reported (Polinski et al., 2014b) suggesting that the timing of PZQ administration possibly needs to be re-considered. We suggest that PZQ should possibly be administered as soon as *Cardicola* spp. DNA is detected in the fish, hence the importance of implementing the use of qPCR during routine checks in the PBT industry and for those checks to be more frequent during early PBT sea-cage rearing. Sea-cage cultured PBT naturally infected with *C. orientalis* and *C. opisthorchis* showed an immune reactivity at early-life stages suggesting that an early immunisation strategy (e.g. vaccine for injection or immersion) might be able to protect the young fish against primary infection.

In humans, and other mammals, helminths or blood flukes are known to regulate the host immune response to promote their own persistence and life span (Hewitson et al., 2009). A key feature of these parasitic helminths is the suppression of the specific immune host response, in particular their ability to make the host T-helper cells unresponsive by inducing anti-inflammatory molecules (Hewitson et al., 2009). Although several studies on blood fluke related immunopathologies have been conducted in mammals, fish parasitoses are poorly understood. A study on serum antibody and complement activity in *Cyprinus carpio* experimentally infected with the blood fluke *Sanguinicola inermis* investigated the role of host-like molecules in immune evasion (Roberts et al., 2005); however it did not provide evidence that the parasite evades the host immune response. Thus, whether

mechanisms to evade the fish host immune system exist and are used by blood fluke species is currently unknown.

The gene expression results presented in this study indicate an involvement of cellular markers of immune cells, particularly antigen presenting cells (MHC-I), B cells (IgM), T cells (TCR-β), and classic inflammatory signalling cytokines (IL-1β), in the gills of farmed juveniles PBT naturally infected by the blood flukes *C. orientalis* and *C. opisthorchis*. In particular, the strong induction of the MHC-I and IgM mRNA expression in the gill during *Cardicola* spp. infection indicates an immunoreaction to the parasite.

In the present study IgM was strongly up regulated in the gills of PBT during late infection with *Cardicola* spp. and most notably in *C. orientalis* infected gills relative to non-infected samples. Since the qPCR detection method used in this research does not distinguish between different *Cardicola* spp. life stages, it was not possible to determine as to which parasitic life stage may have induced the changes in IgM mRNA expression. However, since the IgM mRNA expression increased during late stages of *Cardicola* spp. infection it is possible that late parasitic stages (e.g. eggs and miracidia) rather than early stages, may have induced the up-regulation. In agreement with our results, a strong increase of IgM transcription in PBT gills and heart infected by *Cardicola* spp. during late infection was previously observed (Polinski et al., 2014b). The positive relationship between IgM transcription and *C. orientalis* but not *C. opisthorchis* relative prevalence in the gills is supported by the same results reported previously (Polinski et al., 2014b). This potentially targeted immune response suggests that *C. orientalis* is more immunoreactive than *C. opisthorchis*.

In this study a strong up-regulation of MHC-I at the mRNA level was observed in PBT gills infected by *Cardicola* spp. relative to non-infected gill samples and its induction was much stronger during late stages of infection. As in the case of IgM up-regulation, MHC-I induction during late infection may be elicited by late parasitic stages. This finding suggests that the parasites triggered the initiation of the host immune response. In fact, the main function of the major histocompatibility (MH) receptors is to present foreign antigens to the T cells (Alvarez-Pellitero, 2008a). Moreover, for

the first time we observed that the increased MHC-I mRNA expression was only in gill samples infected by *C. orientalis* relative to uninfected samples. This result is further supported by our observation of a positive correlation between the MHC-I transcription and *C. orientalis* relative prevalence in PBT gills. According to our knowledge, this is the first report of differential MHC-I gene expression in PBT gills infected by blood flukes. The up-regulation of MHC-I has been reported in other parasitic infections in fish (Chang et al., 2005; Fast et al., 2006; Kania et al., 2007; Pennacchi et al., 2014).

Little is known about the expression of genes encoding TCRs in fish parasitosis. In the present study, the increase of TCR-β gene expression observed during late stages of *Cardicola* spp. infection suggests an activation of T cell receptors and in general a recruitment of T cells to the infection site, the gill. In fact, we know that TCRs are present on the surface of T lymphocytes where they bind specifically to foreign antigens presented by peptide-MHC complexes (Nakanishi et al., 2011). Furthermore, TCRs are heterodimers consisting of either α/β and γ/δ subunits combinations, which have been described in teleosts (Alvarez-Pellitero, 2008a). In contrast to our results, Polinski and colleagues did not observe significant changes in the expression of TCR-β in the gills of PBT infected with *Cardicola* spp. even though they detected an increase in the heart during late infection (Polinski et al., 2014b). One reason for the difference between the two studies may be that the time points investigated in our study included some earlier ones during infection (Polinski et al., 2014b). Thus, the previous work may have missed detecting the increase of TCR-β. Furthermore, the highest TCR-β increase (3-fold) detected was at 49 DPT while the previous study focused more on late infection with fewer time points investigated (Polinski et al., 2014b). Another reason for the difference between the two works may be due to the different infectious pressure of the two *Cardicola* spp. as a result of annual variability and different fish cohort which may have influenced the gene expression results in the gills (Polinski et al., 2014b). An up-regulation of TCRs gene expression has been reported also in previous research on fish parasitosis and in other infectious diseases affecting fish (Ingerslev et al., 2009; Nithikulworawong et al., 2012; Overgard et al., 2012; Pennacchi et al., 2014).

In this research the pro-inflammatory cytokine interleukin-1β showed a transient increase of its expression during early infection (28 DPT) with *C. orientalis* in the gills of PBT. This finding suggests that the parasitic infection resulted in an inflammatory process in the host gills, possibly caused by immature or developing worms in the gills. IL-1β has been shown to play an important role during infection by acting as a mediator of inflammation in fish (Rauta et al., 2012). Similarly, infection of PBT ranched in Mexico with a didymozoid, *Didymocystis wedli,* resulted in an up-regulation of IL-1β in the infected gills relative to uninfected controls (Mladineo and Block, 2010).

In contrast to our results Polinski and colleagues observed no significant changes in the expression of IL-1β at the mRNA level in PBT gill, suggesting that *Cardicola* spp. infection failed to elicit the inflammatory response in the host (Polinski et al., 2014b). The difference between these two studies is most likely due to the fact the latter focused on late infection and that the earliest time point analysed for gene expression was at 66 DPT, by which time the PBT were already co-infected by both *C. orientalis* and *C. opisthorchis* late life cycle stages (Polinski et al., 2014b). We observed the increased transcription of IL-1β only at the time where co-infection with *C. opisthorchis* was not present. Furthermore, the detected increase was relative to a time point (35 DPT) when the PBT were infected only by *C. opisthorchis,* suggesting that most likely *C. orientalis* elicited the up-regulation of IL-1β mRNA expression in the gills. Since both microscopy and histopathology showed no sign of *Cardicola* eggs in the gills at the same time point (28 DPT) most likely the DNA detected belonged to *C. orientalis* cercaria and/or adult life stages. Thus, it is possible that the cercariae entering the gill caused the host inflammatory response.

The gene expression results herein reported are consistent with previous literature on host response to various fish parasitosis. Especially, the up-regulation of IgM has been reported during the amoebic gill disease (AGD) in Atlantic salmon gill (Pennacchi et al., 2014), infection with the ciliate *Ichthyophtirius multifiliis* in rainbow trout head kidney and skin (Sigh et al., 2004a), infection with the crustacean *Argulus siamensis* in rohu head kidney (Kar et al., 2015), and salmon louse infection in Atlantic salmon skin (Tadiso et al., 2011). An increase of MHC-I transcription was observed during

infections with *N. perurans* in Atlantic salmon gills (Pennacchi et al., 2014), the copepod *Lepeophteirus salmonis* in Atlantic salmon head kidney (Fast et al., 2006), the monogenean *Gyrodactylus salaris* in Atlantic salmon skin (Kania et al., 2007), and the copepod *Sinergasilus major* in the grass carp liver (Chang et al., 2005). The expression of TCR-α increased in AGD-affected gills of Atlantic salmon (Pennacchi et al., 2014) and in the gill, spleen, liver, and head kidney of Atlantic salmon infected with the infectious pancreatic necrosis virus (Ingerslev et al., 2009). Furthermore, TCR-β expression has been showed to increase during viral and bacterial infections, particularly in Atlantic halibut brain and eye infected with nodavirus (*in vitro*) (Overgard et al., 2012) and in Nile tilapia spleen infected with *Streptococcus sagalactiae* (see Nithikulworawong et al., 2012). An up-regulation of the IL-1β expression was reported during infection with *I. multifiliis* in rainbow trout skin, head kidney and spleen (Sigh et al., 2004a) and in carp skin (Gonzalez et al., 2007), *N. perurans* in rainbow trout (Bridle et al., 2006b) and Atlantic salmon gills (Bridle et al., 2006a; Loo et al., 2012; Morrison et al., 2007; Morrison et al., 2012; Pennacchi et al., 2014; Young et al., 2008a), *Trypanoplasma borreli* in carp head kidney (Saeij et al., 2003), and *Myxobolus cerebralis* in rainbow trout skin (Severin and El-Matbouli, 2007).

In this study, the *Cardicola* spp. relative prevalence results confirmed the importance of using the quantitative and species specificity of the qPCR to map the parasitic DNA in the fish host and to further understand the infection dynamic. In agreement with Polinski and colleagues (Polinski et al., 2014b), we observed high quantities of *C. orientalis* DNA in heart samples during late infection. This high quantity detected in the heart during late infection may be related to a specific stage of the parasite life cycle, possibly to the eggs delivery in the host blood system. In the gills, *C. orientalis* DNA was first detected during early infection (at 21 DPT) and, although not as sensitive as the qPCR technique, the first observation of eggs at 69 DPT both with histology and microscopy implies that this DNA most likely belongs to immature or developing worms. This finding also suggests that fish gills are the entry site of *C. orientalis* into the host. The *C. opisthorchis* DNA observed in the heart and gills during early infection, before our observation of adult flukes in the heart and eggs in the

gills, could be associated with the cercaria stage infecting the fish host. Thus, this means that both *Cardicola* spp. infecting PBT use the gills as entrance to the host body. In contrast to Polinski and colleagues (Polinski et al., 2014b), we first observed *C. opisthorchis* DNA 31 days earlier in the gill and 25 days earlier in the heart whereas *C. orientalis* DNA first appearance in both organs showed a similar onset of infection to their previous study (Polinski et al., 2014b). Although *C. forsteri* has been recently reported in PBT and a terebellid (intermediate host) (Shirakashi et al., 2016) none of the PBT samples examined in this study contained *C. forsteri* DNA. However, C. *forsteri* cercarie were observed only in one terebellid specimen out of 13 *Cardicola* infected specimens and in a single PBT (Shirakashi et al., 2016). This previous report together with our observations suggest that *C. forsteri* presence in PBT may be sporadic and/or occasional. Future routine checks preferably using molecular detection techniques will clarify *C. forsteri* distribution, dynamics and timing of infection.

The inflammatory reaction observed in histological sections of PBT gills infected with *Cardicola* spp. eggs was consistent with the histopathological results reported in a previous study (Shirakashi et al., 2012b). Furthermore, a similar inflammatory response was described in SBT gills infected with *C. forsteri* (see Colquitt et al., 2001) and in ABT infected with an unknown blood fluke species (Ruiz de Ybanez et al., 2011). In this study, the light pathology associated with *Cardicola* spp. eggs in the gills is mostly associated with *C. opisthorchis*. In fact, at the same time point we detected a much higher prevalence of the latter species DNA in the host gills.

A marked decrease of both *Cardicola* spp. DNA within host heart was observed approximately 24 days post PZQ administration to PBT in sea-cage. However, given the qPCR inability to distinguish between live and dead parasites there is the possibility that the *Cardicola* DNA detected post PZQ treatment belonged to dead parasites. In fact, the *Cardicola* DNA longevity, following the parasite death, in the host is unknown and needs investigation. In general, our observations for the heart are in agreement with previous results which showed that PZQ is effective on *Cardicola* adults within several days post-treatment (Shirakashi et al., 2012a). Otherwise, in the host gills only *C. opisthorchis* DNA showed a slight reduction whereas *C. orientalis* DNA increased.

In agreement with our findings Polinski and colleagues observed a reduction of both *Cardicola* spp. DNA in the PBT heart 28 days post PZQ treatment, however in comparison to our study they also detected a reduction of both species DNA in the gills (Polinski et al., 2014b). In this research the expression of immune-associated genes, especially IgM and MHC-I, was significantly increased a few days post PZQ treatment. It has been demonstrated *in vitro* that stimulation of PZQ in SBT blood culture and intestinal explants induces immune transcription, including MHC-I and IgM (Polinski et al., 2014a). Thus, we cannot exclude the possibility that the administration of PZQ might have enhanced the PBT immune response inducing the up-regulation of the immune-related genes herein observed.

In conclusion, the results obtained in this study showed that *C. orientalis* and *C. opisthorchis* make their appearance in the PBT earlier than previously reported suggesting a potential benefit of earlier PZQ administration. This study also highlights the importance of implementing qPCR techniques for PBT health checks to determine the exact onset of infection which may be different every year. Future studies on *Cardicola* infections should also aim to develop molecular detection techniques capable of distinguishing between different life stages of the parasites to improve our knowledge of the infection dynamics. At the transcriptional level, *C. orientalis* and *C. opisthorchis* infections increased the expression of genes involved with the inflammatory and immune response in the gills of PBT early life stages. In particular, part of this response appeared to be directed toward *C. orientalis* infection. However, although the host showed immune responsiveness to *Cardicola* spp. primary infection the response seemed ineffective at mitigating the infection, hence the necessity of PZQ treatment. This immune reactivity detected at early life stages may be further exploited to develop an immunisation strategy which would allow the PBT industry to apply an early intervention strategy by injection or immersion vaccination.

CHAPTER 5

GENERAL DISCUSSION

This research project focused on investigating the host immune response to the parasites *N. perurans* and blood flukes from the genus *Cardicola* which represent significant threats to the health of two commercial fish species commonly farmed in sea-cages, Atlantic salmon and Pacific bluefin tuna (PBT). In Chapter 2 and Chapter 3 the Atlantic salmon inflammatory and immune response to *N. perurans*, the aetiological agent of AGD, was investigated at the gene expression level following a single (Chapter 2) and multiple (Chapter 3) experimental exposures to the parasite. In Chapter 4 the transcription of inflammatory and immune-related genes was examined in juvenile PBT naturally infected by the blood fluke species *C. orientalis* and *C. opisthorchis*. Additionally, in the same chapter the parasite specific relative presence in PBT main affected organs and the natural infection timeline were described by using a recent species-specific molecular detection technique. Overall, my findings provide new insights into the host-pathogen interaction in parasitoses of marine cage-cultured fish and could contribute to improving the fish health in sustainable mariculture providing the bases of knowledge necessary to the development of future immunostimulatory treatments. Finally, these findings further our understanding of the fish response against parasitic infections which cause extensive morphological alterations of the gill structure (e.g. fusion of lamellae, epithelial hyperplasia, an increase in the number of mucous cells) typical of a proliferative condition.

This thesis gives new insights into the immune response to *N. perurans* and *Cardicola* spp. during early infection at the gene expression level and, for the first time, provides evidence that a classical inflammatory response is triggered in the gills of Atlantic salmon infected with *N. perurans* and of PBT infected with *Cardicola* species. In particular, my results suggest that this inflammatory response is elicited during early primary exposure to the parasites and, for AGD, it is likely that the amoebic burden plays a critical role determining the power of this response. Following a single early exposure to *N. perurans* all the inflammatory (IL-1β and TNF-α) and immune (TCR-α chain, CD8-α, CD4-α, MHC-I, MHC-II, IgM and IgT) associated mRNA transcripts of Atlantic salmon investigated were significantly up-regulated in response to the parasite 10 days post-exposure (Chapter 2). Most notably, my results provided evidence of the involvement of a cellular immune

response and infiltration of CD8+ T-cells, antigen presenting cells and B-cells in the AGD-affected gills of the host. The immune response occurred in particular in the "normal" tissues at the periphery of the AGD-lesions of the AGD-affected gills. Furthermore, these findings suggest the possible involvement of a host antibody response. Four (IgM, MHC-I, TCR-β and IL-1β) of the six (IL-1β, IL-8, IgM, TCR-β, MHC-I and MHC-II) immune associated mRNA transcripts of bluefin tuna analysed for gene expression were significantly increased (time points: 28, 42, 49, 56, 69, 77 DPT) in the gills of PBT naturally infected by *C. orientalis* and *C. opisthorchis* (Chapter 4). In *Cardicola* infection, this up-regulation of immune-related genes was consistent with the structural changes in the gills (e.g. granulomatous response, increase in the numbers of mucous cells, epithelial hyperplasia, lamellar fusion).

In mammals, helminth species such as those belonging to the genus *Schistosoma* and the amoeba *Entamoeba histolytica* are known to suppress the host inflammatory response to benefit their life span or facilitate the infection (Ghosh et al., 1995; van den Biggelaar et al., 2000). For both amoebae and schistosomes infecting mammals, the induction of the anti-inflammatory cytokine IL-10, which is able to suppress inflammation and T-helper cell responses, seems to be a key factor for their success allowing a long lasting persistence in the host (Soboslay et al., 2006; van den Biggelaar et al., 2000; Walsh et al., 2009). This suppression of inflammation by blood flukes has been suggested in a previous study on PBT (time points investigated: 22, 39, 66, 85, 113 DPT) (Polinski et al., 2014b). In contrast, my research showed the up-regulation of the pro-inflammatory cytokine IL-1β in the gills of PBT infected by *Cardicola* species 28 DPT (Chapter 4). Interestingly, this increase of the inflammatory response seems to be caused by *C. orientalis* rather than *C. opisthorchis* and only during early infection, as confirmed by specific detection by qPCR (Chapter 4). In agreement with previous research (Polinski et al., 2014b), our results showed that although the host is capable of mounting an inflammatory and immune response to the parasite. This response does not seem to clear or mitigate a primary infection requiring the oral administration of the anthelmintic drug praziquantel (PZQ) to clear the parasitic infection.

Immunosuppression has been also suggested in AGD during late infection in Atlantic salmon (25 days post-exposure) where a down-regulation at transcriptional level of the antigen presentation pathways, particularly MHC-I and –II, was described (Young et al., 2008a). This is in agreement with the results reported in Chapter 3, where following reinfection (137 days post first exposure and 24 post last exposure) and a long-term exposure (24 days post-exposure) to *N. perurans*, some decrease in the host immune response at gene expression level was observed in the gills of AGD-affected Atlantic salmon. However, it is important to highlight that this general decrease of the immune response has been observed in association with an amoebic concentration of 500 cells/L in the study of Young et al., 2008a, 475 cells/L in the long-term trial and 194, 158, 1000, 475 cells/L in the reinfection trial (Chapter 3). The induction and role of IL-10 in the anti-inflammatory response to *N. perurans* and *Cardicola* species still needs to be confirmed and represents an interesting aspect to investigate in future research.

Altogether, these findings on the inflammatory/immune response in AGD and *Cardicola* infection, especially in the early infection, imply that the host might be able to mount a protective cell-mediated immune response against the parasites. Therefore, these results are of utmost relevance for the potential development of immunostimulant treatments to be implemented in the farming of Atlantic salmon and PBT to strengthen the existing host cell-mediated immunity. Immunostimulants can be organic, inorganic or synthetic products, such as probiotic bacteria, levamisole, glucans and some vitamins and hormones, that have a boosting effect on the innate immunity.

There are a few studies available on attempts to boost the Atlantic salmon immune response to *N. perurans* (see **Table 5.1.**), whereas there is only one field study that investigated the effects of immunostimulants on bluefin tuna immune response, although not particularly against *Cardicola* (see Kirchhoff et al., 2011). In this study the authors used baitfish supplemented with vitamins (mostly E and C) or vitamins and immunostimulants (nucleotides and β-glucans) to enhance the immune response, health and performance of ranched adult *Thunnus maccoyii* (Southern bluefin tuna) (average fork length 95 cm) (Kirchhoff et al., 2011). The authors detected an enhancement of the

lysozyme activity in the vitamin supplementation groups relative to control (Kirchhoff et al., 2011). However, performance (i.e. survival, condition index and crude fat), health (i.e. blood plasma variables including pH, osmolality, cortisol, lactate and glucose) and alternative complement activity were not significantly improved in any of the supplemented diet groups (Kirchhoff et al., 2011). Improvements in performance through vitamin supplementation including survival, *C. forsteri* prevalence and intensity, and alternative complement activity were observed although they were tow-specific (Kirchhoff et al., 2011). In particular, at the end of the trial (19 weeks of ranching), the *C. forsteri* prevalence was significantly lower in the vitamin-fed group relative to control and immunostimulant-fed group in one of the two tows (Kirchhoff et al., 2011). One reason for this limited effect on the *Cardicola* prevalence and fish immune response may be related to the timing/method of administration and the fish age. This previous attempt of using immunostimulants in bluefin tuna diet suggests that intervention may be easier on early bluefin tuna life stages held in tanks (farm raised). In fact, this would allow to experiment with other methods to administer immunostimulants such as injection and immersion before transferring the fish to the sea-cages. Furthermore, knowing the life-history of the fish might help to explain any differences detected in administration method/timing and type of immunostimulant used.

To date, all the previous attempts to boost the fish immune system to prevent AGD (see **Table 5.1.**) had no or limited effect on protection, hence the total reliance of the industry on the FW and hydrogen peroxide bathing of the AGD-affected fish as a mitigation strategy. Only the injection of CpG-ODNs (oligodeoxynucleotides containing cytosine-phosphodiester-guanine) (Bridle et al., 2003) and two experimental diets (Dick, 2012) produced some encouraging results, 38% increase in protection and 27% increase in survival respectively. CpG-ODNs inclusion in feed (2 mg kg^{-1}) has also been reported to successfully induce an inflammatory response, as measured by the expression of IL-1β (13 and 26 days post-exposure) and matrix metalloproteinase (MMP)-9 (6 and 13 days post-exposure) in the head kidneys of Atlantic salmon infected with sea lice (*Lepeophtheirus salmonis*) and to significantly reduce cortisol levels relative to infected fish on control diet (Poley et al., 2013).

Furthermore, another study on sea lice in Atlantic salmon showed that CpG-ODN fed fish had decreased (31-46%) infection levels when compared to the uninfected control group (Covello et al., 2012). An increase of the inflammatory response due to CpG-ODN treatment (via injection) has been observed also in gilthead sea bream (*Sparus aurata*) head-kidney where an up-regulation of IL-1β was observed (Cuesta et al., 2008).

From these previous studies (see **Table 5.1.**) it seems likely that the type of administration is relevant to obtain an effective boost of the fish immune system since the only treatment that showed a marked increase in protection was administered via intraperitoneal injection (Bridle et al., 2003). Although immunostimulants may be administered by injection, immersion or orally, the oral administration represents the most common strategy as it is less time consuming, less stressful to the fish, cheaper and easier to administer (Yanong, 2009). Two downsides of orally administered immunostimulants are that it conveys relatively short immunostimulation compared to the other strategies and that it needs to be well designed to pass intact through the fish intestine to properly stimulate the immune system (Yanong, 2009).

IMMUNOSTIMULANT	APPLICATION	CHALLENGE	REFERENCE
Betaglucans	Oral	Cohabitation	(Zilberg et al., 2000)
Levamisole	Oral	Cohabitation	(Zilberg et al., 2000)
CpG-1668 in PBS (50 µg/fish)	Injection (i.p.)	6 days later 2460 cells/L	(Bridle et al., 2003)
Betaglucans (diet A 1%, diet B 1%, diet C 0.3%)	Oral	7 days later 1150 cells/L	(Bridle et al., 2005)
Betaglucan enriched with other components ecoboost	Oral	458 cells/L	(Nowak et al., 2004)
Aquacite and betabec (betaglucans, bioflavenoids and vitamins)	Oral	Natural infection on farm	(Powell et al., 2007)
Experimental immunostimulant containing diets	Oral	500 cells/L	(Dick, 2012)

Table 5.1. Experimental immunostimulant treatments used to prevent AGD in Atlantic salmon (modified from Nowak et al., 2014).

Betaglucans are carbohydrates found in the cell walls of some plants, yeast, bacteria, seaweed, and fungi, and are recognised by receptors on fish monocytes/macrophages (Dowling et al., 1992; Engstad and Robertsen, 1993). Furthermore, betaglucans have been widely used to develop immunostimulant diets or treatments for various fish bacterioses due to their ability to stimulate fish innate immunity (Ai et al., 2007; Kumari and Sahoo, 2006; Sahoo and Mukherjee, 2002; Sirimanapong et al., 2015). However, their inclusion in treatments to boost the fish immunity did not show promising results against *N. perurans* (see **Table 5.1.**) or to improve health of bluefin tuna (Kirchhoff et al., 2011). This may be due to different reasons such as sub-optimal feed inclusion rates, diet and betaglucan formulations, administration timing and type. Betaglucans have never been used as immunostimulants in PBT against *Cardicola* infection, therefore it would be interesting to test their effects on early rearing.

Probiotics are therapeutic preparations containing live bacteria or yeast that supplement the normal gastrointestinal flora, and they have been shown to act as immune-modulators providing protection against fish bacterioses (Nayak, 2010). In rainbow trout the administration of a diet enriched with probiotics produced an increase of the serum lysozyme and complement activities and the head kidney leucocytes' phagocytic activity (Panigrahi et al., 2004). Furthermore, an up-regulation of the cytokines IL-1β, TNF-1 and -2 and transforming growth factor (TGF)-β was detected in the fish spleen and the head kidney in a similar study (Panigrahi et al., 2007). Although, more investigation needs to be done to verify the applicability and use of probiotics in aquaculture, they represent ideal candidates as immunostimulants against fish parasitoses.

This thesis has shown that most likely the timing of treatment administration relative to the infection is fundamental for a proper stimulation of the immune system during fish parasitoses. The detected inflammatory/immune response during early infection highlights the importance to focus the intervention earlier in both AGD and *Cardicola* infection. At this stage, the main obstacle to the development of immunostimulant strategies to prevent *Cardicola* infection outbreaks is the lack of an experimental model for *Cardicola*.

This PhD thesis further confirms the importance of using molecular techniques to quantitatively and specifically detect *Cardicola* DNA in cage cultured bluefin tuna, providing new insight into the infection dynamic and distribution of *C. orientalis* and *C. opisthorchis* in PBT gills and heart (Chapter 4). The qPCR was confirmed a highly reliable technique, relatively to gill microscopy and heart flushes, to detect the specific parasite DNA allowing farmers to determine the exact onset of infection. This would also help to decide the appropriate timing for immunoprophylactic strategies. Furthermore, for routine checks on the fish farm, this technique can be performed using blood samples and is therefore non-lethal (Polinski et al., 2013a; Polinski et al., 2014b).

Given the recent discovery of *C. forsteri* in farmed PBT (Shirakashi et al., 2016) further research is needed to investigate the distribution of this third species in the host and its infection dynamic. Nevertheless, in my research *C. forsteri* DNA was not observed in PBT gill and heart samples. This suggests that the presence of this species might be sporadic and/or occasional in the host depending on the occurrence of appropriate environmental conditions and/or availability of the intermediate host nearby the sea-cages. Therefore, in future routine monitoring of *Cardicola* infection the farmers should look for all the *Cardicola* species found to infect PBT by using molecular detection techniques that can distinguish between different species.

In a previous *in vitro* experiment, stimulation with PZQ in bluefin tuna blood cell culture and intestinal explants has been shown to induce a transcriptional response of selected immune-related genes, especially IgM, MHC-I and TCR (Polinski et al., 2014a). This finding suggests an immunostimulatory activity of PZQ. Previous research on PZQ showed that usually the drug is cleared from the fish body within 24 h (Ishimaru et al., 2013). Therefore, in my research, the possibility that the detected increase of the immune response post PZQ treatment might be due to the stimulation of the host immune system by PZQ, even if a few days after the administration of the drug, should be taken under consideration. Unfortunately, the lack of negative control (fish not treated with PZQ) made it impossible to know if the administration of PZQ had a key role at boosting the

PBT immune system. Further investigation will be necessary to assess *in vivo* the effects of PZQ on the PBT immune system and its potential application as an immunostimulatory treatment against *Cardicola* infection. Nevertheless, a significant limiting factor for research on bluefin tuna is the high economic value that the farmers give to each farmed fish and the consequent difficulty at sampling enough individuals for investigation. Furthermore, very recently the IUCN updated the status of PBT, *Thunnus orientalis*, in the red list from "Least Concern" to "Vulnerable" which will make future research on this fish species even harder (IUCN, 2014). In light of this the farming of PBT and its health management might play a key role for the future survival of this species.

The current research highlights the relevance of describing the sample composition (e.g. percentage of lesion in the sample) in gene expression studies on the fish immune response against AGD (Chapter 2). The ratio of normal to hyperplastic gill tissues in samples from AGD-affected gills determined the strength of the immune signalling detected (Chapter 2). This finding was fundamental to understand why the previous studies on gill gene expression of Atlantic salmon during AGD reported a down-regulation of AGD-lesion samples relative to non-lesion samples in the AGD-affected gills (Loo et al., 2012; Morrison et al., 2006a; Morrison et al., 2012; Wynne et al., 2008a; Young et al., 2008a). In fact, AGD-lesions are mainly constituted of proliferating epithelial cells which do not express the majority of immune genes. Therefore, it is most likely that the hyperplastic epithelia component of the AGD-lesion samples resulted in the lack of immune signalling (Nowak et al., 2013; Nowak, 2012). Unfortunately, an approach similar to the one used in Chapter 2 (i.e. biopsies composed of a ratio of infected/uninfected gill tissue) was not possible for PBT. Differently from AGD-affected gills, signs of *Cardicola* infection pathology (e.g. *Cardicola* eggs and/or adults) in the gills of PBT early life stages are not grossly visible. Apart from histology, the visualisation of *Cardicola* eggs but not adults in the gills is possible only by transferring a few gill filaments to a slide and observing it using optical microscopy.

Based on the biopsy severity results obtained in Chapter 2 (small % of AGD-lesion in the biopsy = stronger immune signalling), in the resistance and long-term trial in Chapter 3 the gill

biopsies were not assessed for AGD-severity assuming that targeting normal/healthy gill tissue, with a small component of AGD-lesion would have produced a similar immune signalling. Interestingly, the research in Chapter 3 showed that there is little change in the host immune response and a general variability at gene expression level. These findings were observed following reinfection and a long-term exposure to the amoeba and using a lighter amoebic load compared to the challenge of Chapter 2. Furthermore, these observations might be the result of changes in ratio and type of cell populations in the host gills during AGD.

These changes at the cellular level might be caused by an infiltration of different cell populations or altered differentiation of stem cells in the AGD-affected gills (Nowak et al., 2014). The variability at gene expression level is likely due to issues related to sample collection from a heterogeneous organ as the gill which is constituted of different tissues. Indeed, it has been shown that differences between samples at cellular level contribute to data dispersion making difficult the detection of small changes at gene expression level (Disset et al., 2009). Moreover, AGD-lesions, which go through a remodelling/repairing process during the disease progression (Adams and Nowak, 2004), make the gill composition at cellular level even more complex. In this thesis, histological observations showed that also in the gills of *Cardicola* infected PBT, similarly to AGD-affected gills, there is a high cellular heterogeneity (e.g. numerous mucous cells, presence of epithelial hyperplasia and lamellar fusion) between infected and uninfected sites in the same gill section, typical of a proliferative condition (Chapter 4). Overall, the heterogeneity has to be considered in all the gene expression studies on diseases that modify the cellular composition of the organs. Another aspect to consider is that disease is a dynamic process and at a certain time point post-infection, especially during a long-term infection trial, the infection progression between individuals might be different contributing to an increase in gene expression variability.

In future gene expression studies the cellular composition of gill samples should be assessed to further improve our understanding of the host response during AGD and *Cardicola* infection and solve the issue related to sample structural heterogeneity. A technique named tissue compartment

analysis (TCA) might represent a possible solution to the sample heterogeneity issue (Disset et al., 2009). This technique would allow to quantitatively determine the fractional volume of the different structures in the samples by comparing the mRNA expression level of specific markers in pure isolates to the whole sample (Disset et al., 2009). The TCA would be useful to localise the expression site of the target genes in the AGD-affected gill sample. Another sensitive technique that could be used to localise specific mRNAs within tissue samples by using RNA probes, is the RNA *in situ* hybridization (ISH) (Lee et al., 2013). Unfortunately, due to time and technical constraints it was not possible to use these techniques, especially the latter, in this thesis.

Previous challenges with *N. perurans* have shown that the amoebic burden plays an important role at determining the onset and the extent of clinical signs of AGD, especially the appearance of lesions on the gills (Nowak et al., 2014). For instance, usually 500 cells/L generate small AGD-lesions in a few days from first exposure of the fish to *N. perurans* whereas 2500 cells/L cause the insurgence of severe AGD-lesions in 24 h (Nowak et al., 2014). In this thesis, exposing the fish to 2000 cells/L has shown a strong inflammatory response in the AGD-affected gills 10 days post exposure (Chapter 2) whereas following reinfection with significantly lower doses of the parasite the host immune response has shown only little change (Chapter 3). In a recent study on AGD, the authors challenged the fish once using two different amoebic loads, 500 cells/L and 5000 cells/L, and observed the effects on the host immune response at transcriptional level in the affected gills 21 days post-exposure (Benedicenti et al., 2015). In general, they found the cytokines related to Th1, Th17 and T-reg pathways significantly decreased in gill samples from fish challenged with the higher amoebic load (5000 cells/L) and Th2 related cytokines increased in gill samples from fish exposed to both amoebic loads (**Table 5.2.**) (Benedicenti et al., 2015). Unfortunately, in that paper no details were provided about the gill samples composition of the AGD-affected fish (e.g. presence of AGD-lesions in the samples) making hard a comparison with our results and understand the effects of each amoebic load on the pathology and the host immune response. Furthermore, the differences between the two amoebic loads were not statistically investigated at transcriptional level but each amoebic burden was

only related to uninfected control. Therefore, the effects of different amoebic burden (e.g. light versus heavy) on the host transcriptional immune response still need investigation in order to be fully understood. See **Table 5.2.** for a list of the effects at gross, histopathological and gene expression level of experimental infections with different loads of *N. perurans* in Atlantic salmon gills.

In parasitic infections of mammals, proteases (enzymes that performs proteolysis) derived from parasites are thought to be important factors for establishing infection (Yang et al., 2015). The possibility that extracellular products generated by *N. perurans* might have influenced the host immune gene expression should also be taken into consideration and investigated in future studies. A positive relationship between virulence and protease expression has been reported for pathogenic and non-pathogenic amoebae species infecting mammals (Serrano-Luna et al., 2013). Of great interest is the fact that proteases produced by amoebae have been shown to affect the immune system components (e.g. immunoglobulins, complement, and cytokines), mucins and different tissues and cells of the host (Serrano-Luna et al., 2013). To date, there is a lack of information on the presence, identity and role of proteases for *N. perurans*. However, previous authors suggested that *Neoparamoeba* spp. derived cytolytic products might represent the cause of cytopathic effects observed *in vitro* in cultured gill epithelial cells of Atlantic salmon (Butler and Nowak, 2004). A recent work on the loss of virulence of *N. perurans* during clonal culture, showed that an extracellular product produced by the wild type amoeba is associated with the cytopathic effect observed on a salmon cell culture (Bridle et al., 2015). Furthermore, the same authors suggested that this extracellular product produced by the wild type amoeba is most likely a virulence factor associated with AGD (Bridle et al., 2015). Therefore, it would be interesting to investigate the possible generation of proteases or other extracellular compounds by *N. perurans* and if so their role in the host immune system as might be relevant for the development of new therapeutic targets to treat AGD.

Serine proteases are digestive proteases, enzymes that cleave peptide bonds in proteins, and the major part of them in helminthic infections of mammals are associated with metabolic food

processing or penetration of the host tissues (Yang et al., 2015). Other serine proteases have also been showed to play a role in evasion of the host immune system (Dzik, 2006). For instance, in mammals a cercarial elastase (SmCE) with a chymotrypsin-like activity produced by the helminth *Schistosoma mansoni,* it has been reported as a major histolytic protease involved in host skin invasion (Yang et al., 2015). Additionally, previous research reported that proteases produced by helminths may aid the parasites to evade the host immune response by degrading immune effectors or modulating the cellular immunity (Yang et al., 2015). In particular, in mammals it has been showed that in *S. mansoni* infection an elastase-like serine protease helps the parasite to evade IgE-mediated protective response (Pleass et al., 2000). The investigation of the presence of *Cardicola* spp. proteases involved in host invasion and evasion of the immune system in PBT would potentially offer the bases for the development of novel health strategies to prevent the infection. These strategies may contribute to the development of specific drugs/vaccines/therapeutic treatments that disrupt these potential invasion mechanisms and/or inhibit the degradation of the immune pathways caused by the parasites.

Day post-exposure	*N. perurans* cells/L	Up-regulated genes	Down-regulated genes	Gross observations and histopathology	References
0	500	C type lectin complement C3	Peroxiredoxin complement C2	No gross lesions	(Morrison et al., 2006a)
2		C type lectin CC chemokine IgLH		No gross lesions. Hyperplastic epithelial cells leading to fusion of lamellae.	(Morrison et al., 2006a)
5		C type lectin CC chemokine DRTP		No gross lesions. Fusion of several lamellae.	(Morrison et al., 2006a)
8		TCR-α chain DRTP selenoprotein p	MBP-C ortholog LPB NADPH oxidase cytosolic protein p40 phox LPS binding protein	Presence of gross lesions. Extensive fusion of lamellae.	(Morrison et al., 2006a)
10	2000	TCR-α chain CD8 CD4 MHC-I MHC-II α IgM IgT IL-1β TNF-α		Presence of numerous gross lesions.	(Pennacchi et al., 2014)*
12	500	IL-1β TNF-α COX-2		Presence of a few gross lesions. 2.5% of affected filaments.	(Morrison et al., 2012; Young et al., 2008a)
14	450 (over three consecutive days)	IL-1β Peroxiredoxin		Presence of numerous gross lesions. Widespread lamellar fusion and epithelial hyperplasia.	(Bridle et al., 2006a; Loo et al., 2012)
19	500		DRTP CCAAT glutathione S-transferase glutathione peroxidase thioredoxin NADP transhydrogenase	Presence of gross lesions. Epithelial hyperplasia, edema, and interlamellar vesical formation. 0-85% of affected filaments.	(Wynne et al., 2008a)

21	500 (over 4 h)	IL-4/13A	TGF-β1B	Gill score no different from uninfected control.	(Benedicenti et al., 2015)
	5000 (over 4 h)	IL-4/13A IL-4/13B2	IFN-γ TNF-α3 IL-17 A/F1b IL-17 D IL-22 TGF-β 1B IL-10 A IL-10 B Arginase	Gill score significantly different from uninfected control and 500 cells/L.	(Benedicenti et al., 2015)
24	475		IgT MHC-I MHC-II α	Presence of numerous gross lesions. Mean lesion size 21±8.3, 32.5% of filaments with hyperplastic lesions.	Chapter 3 of this thesis*
25	500	IL-1β IL-8 COX-22	IL1 R1 Tollp MHC-I IRF-1 Beta2m TAPBP IFN-γ	Presence of numerous gross lesions. 15% of affected filaments.	(Morrison et al., 2012; Young et al., 2008a)
36	500	IL-1β IL-8 COX-22	MHC-I α3 MHC-II β IRF-1 Beta2m PA28β TAPBP IcIp IL-1 R1 Tollp	Presence of numerous gross lesions. 33% of affected filaments. Extensive hyperplasia of epithelial like cells, resulting in lamellar fusion.	(Morrison et al., 2012; Young et al., 2008a)
38	500	IL-1β		Presence of gross lesions. 88% of affected filaments.	(Morrison et al., 2007)

Table 5.2. Differential gene expression, gross observations and histopathology in the gills of Atlantic salmon experimentally exposed to different loads of *N. perurans*. * indicates findings of this thesis. Modified from Nowak et al., 2014.

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